MESTRADO EM ONCOLOGIA

ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

Decoding the usefulness of miRNAs as biomarkers in breast cancer patients treated with endocrine therapy

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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DECODING THE USEFULNESS OF MIRNAS AS BIOMARKERS IN BREAST CANCER PATIENTS TREATED WITH ENDOCRINE THERAPY

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2017

Decoding the usefulness of miRNAs as biomarkers in breast cancer patients treated with endocrine therapy

Dissertação de Candidatura ao grau de **Mestre em Oncologia** – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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“Arranja tempo para a amizade, é o caminho da felicidade.

Não corras, não te aflijas.

Só estás aqui de passagem, e é curta a tua visita.

O importante é parar e cheirar as flores.”

Vovô

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RESUMO

Introdução: O cancro da mama é a neoplasia mais frequente e a principal causa de morte por cancro nas mulheres em todo o mundo. Aproximadamente 70% dos cancros da mama são do subtipo luminal e expressam os recetores de estrogénio. As terapias mais comuns e eficazes para as doentes com este subtipo são as terapias endócrinas. No entanto, a eficácia destas terapias é limitada, e cerca de 30-40% das mulheres acabam por ter recorrência da doença. Uma vez que os microRNAs têm sido associados com vários mecanismos de sensibilidade e resistência endócrina, estas moléculas podem servir como biomarcadores preditivos e/ou de prognóstico neste subgrupo de doentes.

Objetivo: O principal objetivo desta dissertação de Mestrado foi investigar se microRNAs que estão desregulados em tumores da mama com resistência endócrina podem ser clinicamente relevantes como biomarcadores preditivos e de prognóstico em pacientes com tumores da mama luminais tratadas com terapia adjuvante endócrina.

Materiais e Métodos: Começou-se por realizar um ensaio de expressão global com o objetivo de identificar microRNAs com expressão diferente entre doentes luminais com e sem recidiva da sua doença após o tratamento com terapias adjuvantes endócrinas. Posteriormente, sete microRNAs - miR-30b-5p, miR-30c-5p, miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p e miR-205-5p - foram escolhidos para validação num maior número de tecidos por RT-qPCR. Seguidamente, o potencial de diagnóstico e de prognóstico destes microRNAs foi avaliado através da construção de curvas de ROC e de modelos de Regressão de Cox, respetivamente. Realizou-se igualmente análise do *status* de metilação por PCR quantitativo específico em DNA modificado por bissulfito de sódio. Por último, analisámos igualmente a expressão de microRNAs em tecidos tumorais primários e metástases correspondentes.

Resultados e Discussão: Após o ensaio de expressão global os microRNAs miR-30b-5p, miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p e miR-205-5p foram selecionados para validação, tendo o miR-30c-5p sido utilizado como controlo positivo. A combinação dos microRNAs miR-182-5p e miR-200b-3p num painel foi capaz de identificar cancro da mama em amostras de tecido com uma acuidade de 95,55%. Adicionalmente, os microRNAs miR-30c-5p, miR-30b-5p, miR-182-5p, bem como o miR-200b-5p foram identificados como preditores independentes de resposta a terapias endócrinas. Além disso, os microRNAs miR-182-5p e miR-200b-3p são marcadores de prognóstico em doentes com tumores luminais após tratamento adjuvante com terapias endócrinas. Verificou-se ainda que os microRNAs miR-30b-5p e miR-200b-3p estão significativamente

mais expressos nas metástases relativamente aos tumores primários correspondentes. Além disso, os nossos resultados sugerem que a menor expressão do miR-200b-3p nos tumores com resistência à terapia endócrina é dependente de outros mecanismos epigenéticos, e não da metilação das suas regiões promotoras.

Conclusões e perspectivas futuras: Os resultados sugerem que um painel específico de microRNAs poderá ser útil na decisão terapêutica em doentes com tumores da mama luminais. No entanto, estudos adicionais, idealmente estudos multicêntricos, são essenciais para a sua validação. Como principal perspectiva futura, pretendemos avaliar a expressão destes microRNAs em biópsias líquidas, de maneira a avaliar o seu potencial como biomarcadores não-invasivos.

ABSTRACT

Introduction: Breast cancer is the most frequent malignancy and the leading cause of cancer death among women worldwide. Approximately 70% of BrCa are of the luminal type, expressing the estrogen receptor. One of the most common and effective adjuvant therapies for this BrCa subtype is endocrine therapy. However, its effectiveness is limited, with relapse occurring in up to 40% of patients. Because microRNAs have been associated with several mechanisms underlying endocrine resistance and sensitivity, they may serve as predictive and/or prognostic biomarkers in this setting.

Aims: The major goal of this master dissertation was to investigate whether miRNAs deregulated in endocrine-resistant breast cancer may be clinically relevant as prognostic and predictive biomarkers in luminal

breast cancer patients treated with adjuvant endocrine therapy.

Material and Methods: We started by performing a global expression assay with the aim of identifying microRNAs differentially expressed between luminal patients with or without breast cancer recurrence after endocrine therapy. Then, seven microRNAs - miR-30b-5p, miR-30c-5p, miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p e miR-205-5p - were chosen for validation using quantitative reverse transcription polymerase chain reaction in a larger set of tissue samples. ROC curves and cox-regression models were constructed to evaluate miRNAs diagnostic and prognostic performance, respectively. DNA methylation analysis was also performed by sodium bisulfite modification followed by quantitative methylation-specific polymerase chain reaction. Furthermore, microRNAs expression levels were also analyzed in metastatic tissues and the paired primary tumor tissue.

Results and Discussion: From the initial global expression assay, miR-30b-5p, miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p and miR-205-5p were selected for further validation, and miR-30c-5p was chosen as a positive control. The combination of miR-182-5p and miR-200b-3p accurately detects BrCa in tissue samples with an overall accuracy of 95.55%. MR-30c-5p, miR-30b-5p, miR-182-5p and miR-200b-5p were found to be independent predictors of clinical benefit from endocrine therapy. Moreover, miR-182-5p and miR-200b-3p displayed independent prognostic value for disease recurrence in luminal BrCa patients after endocrine therapy. MiR-200b-3p and miR-30b-5p were significantly higher in metastatic tissues when compared to the paired primary tumor tissues. Furthermore, our results suggest that miR-200b-3p's downregulation in endocrine-

resistant tumors might be dependent on other epigenetic mechanisms rather than DNA methylation.

Conclusions and future perspectives: We concluded that selected miRNAs may constitute clinically useful ancillary tools for management of luminal BrCa patients. Additional validation, ideally in a multicentric setting, is required to confirm our findings. As a future perspective, we intend to assess these miRNAs expression in liquid biopsies in order to evaluate their potential as non-invasive biomarkers.

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LIST OF ABBREVIATIONS

ABL1 – ABL proto-oncogene 1, non-receptor tyrosine kinase

ACTβ - Actin β

ADH1B - Alcohol Dehydrogenase 1B (Class I), Beta Polypeptide

AGO – Argonaute

AI – aromatase inhibitor

AJCC – American Joint Committee on Cancer

Akt - Protein kinase B

ALCAM - Activated Leukocyte Cell Adhesion Molecule

ANA – Anastrozole

AntiE – Anti estrogen

Ap1 - Activation protein 1

ARPP19 - cAMP-regulated phosphoprotein 19

ASCO – American Society of Clinical Oncology

AUC – Area Under the Curve

BBC3iso-2 - BCL2 Binding Component 3 isoform 2

BCAR4 - Breast cancer anti-estrogen resistance 4

BCL2 – BCL2, apoptosis regulator

BCL2L11 - BCL2 like 11

BMP7 - Bone morphogenetic protein 7

bp – Base pairs

BrCa – Breast cancer

BRCA1 – BRCA1, DNA repair associated

BRCA2 – BRCA2, DNA repair associated

CA 15-3 - Carbohydrate antigen 15-3

CCN – Cyclin

CDH – Cadherin

CDK – Cyclin-dependent kinase

CDKN - Cyclin-dependent kinase inhibitor

CEA - Carcinoembryonic antigen

Cht – Chemotherapy

CI – Confidence interval

CK – Cytokeratins

COL2A1 - Collagen type II alpha 1

CpG - Cytosine-phosphate-Guanine

CTNNB1 - Catenin Beta 1

CYP19A1 - Cytochrome P450 family 19 subfamily A member 1

DCIS - Ductal carcinoma in situ

DFS - Disease-free survival

DGCR8 - Di-George syndrome critical region gene 8

DMFS – Distant metastasis-free survival

DNMT - DNA methyltransferase

DSCAM-AS1 - DSCAM Antisense RNA 1

E_2 – Estradiol

E2F3 - E2F transcription factor 3

EDTA – Ethylenediamine teracetic acid

EFNA3 - Ephrin A3

EGFR - Epidermal growth factor receptor

EMT - Epithelial–mesenchymal transition

ER – Rstrogen receptor

ERBB - Erb-B2 Receptor Tyrosine Kinase

ERE - Estrogen response element

ERFS - Endocrine resistance-free survival

ERK - Extracellular signal regulated kinases

ESMO – European Society for Medical Oncology

ESR1 - Estrogen Receptor 1

ESRRG - Estrogen related receptor gamma

ET – Endocrine therapy

EZH2 - Enhancer of zeste homolog 2

FFPE - Formalin-fixed paraffin-embedded

FGFR1 - Fibroblast Growth Factor Receptor 1

FGFRL1 - Fibroblast growth factor receptor-like 1

FOX - Forkhead Box

FULV – Fulvestrant

G – Grade

GATA3 - GATA Binding Protein 3

GEMIN4 - Gem (nuclear organelle)-associated protein 4

GnRH - Gonadotropin-releasing hormone

GRB7 - Growth Factor Receptor Bound Protein 7
GSTP1 - Glutathione S-Transferase Pi 1
GTP - Guanosine-5'-triphosphate
H&E - Hematoxylin-eosin
HAT - Histone acetyltransferase
HDAC - Histone deacetylase
HDMs - Histone demethylases
HER2 - Human Epidermal growth factor Receptor 2
HMTs - Histone methyltransferases
HNRNPL - Heterogeneous Nuclear Ribonucleoprotein L
HPF – High-power field
HR – Hazard ratio
HRT – Hormone-replacement therapy
HSP - Heat shock proteins
IDC - Invasive ductal carcinoma
IGF-1 - Insulin-like growth factor-1
IGFBP2 – Insulin like growth factor binding protein
IGFR1 - Insulin-like growth factor 1 receptor
IHC - Immunohistochemistry
ILC - Invasive lobular carcinoma
INPP5D – Inositol polyphosphate-5-phosphatase D
IPC - Inter-plate calibration
IRR - Incidence rate ratio
KIT - KIT Proto-Oncogene Receptor Tyrosine Kinase
KLF4 – Kruppel like factor 4
KRAS – KRAS proto-oncogene, GTPase HMGA2 – high mobility group AT-hook 2
KRT – Keratin
LAM – Laminin
LATS2 – Large tumor suppressor kinase 2
LCIS - Lobular carcinomas in situ
Ldbr - lariat debranching enzyme
LNA - Locked Nucleic Acid
lncRNA – Long non-coding RNA
Lum – Luminal

MAGI2 - Membrane-associated guanylate kinase inverted 2
MAPK - Mitogen-activated protein kinase
MBC - Methyl-CpG binding domain
MERKT – MER proto-oncogene, tyrosine kinase
MET - Mesenchymal-to-epithelial transition
MET - MET Proto-Oncogene, Receptor Tyrosine Kinase
MiR – MicroRNA
MiR-200f – MiR-200 family
MiR-30f – MiR-30 family
MiRNA – MicroRNA
MKI67 - Marker Of Proliferation Ki-67
MRI - Magnetic resonance imaging
mRNA – Messenger RNA
MSP – Methylation Specific Methylation
MTDH - Metadherin
MYBL2 - MYB Proto-Oncogene Like 2
MYBL2 - MYB Proto-Oncogene Like 2
MYC – MYC proto-oncogene, bHLH transcription factor
n.a. – not applicable
NBr – normal breast tissues
ncRNAs - Non-coding RNAs
NF-κB - Nuclear factor kappa B
NPV - Negative predictive value
NSAID - Nonsteroidal anti-inflammatory drug
NST - No special type
nt - Nucleotide
OR - Odds ratio
ORF - Open reading frames
OS – Overall survival
P1 – Promoter 1
P2 – Promoter 2
P25 – Percentile 25
PDCD4 – Programmed cell death 4
PGR - Progesterone Receptor

PI3K - phosphoinositide3-kinase

PI3KR1 – Phosphoinositide-3-Kinase Regulatory Subunit 1

POL – polymerase

PPV - Positive predictive value

PR - Progesterone receptor

PRKAA1 – Protein kinase AMP-activated catalytic subunit alpha 1

PTEN - Phosphatase and tensin homolog

qMSP - Quantitative real-time methylation specific PCR

R – Resistance

RAD52 - RAD52 homolog, DNA repair protein

Ran - RAs-related Nuclear protein

RASSF1A - Ras Association Domain Family Member 1

RB1 – Retinoblastoma 1

Rec – Recurrent

RHOA – Ras homolog family member A

RISC - RNA-induced silencing complex

RISC - RNA-induced silencing complex

ROC - Receiver Operating Characteristic

RR -relative risk

rRNAS – ribosomal RNAs

RS - Recurrence score

RT – Radiotherapy

S – Sensitivity

SDS – Sodium Dodecyl Sulfate

SERD - Selective SER down-regulators

SLNB - Sentinel lymph node biopsy

snoRNAs - Small nucleolar RNAs

SOCS - Suppressor of cytokine signaling

SOX2 – SRY-box 2

SRE - Serum response element

SSC - Special subtypes carcinomas

TCL1A – T-cell leukemia/lymphoma 1A

TGFBR1 - Transforming growth factor, beta-receptor 1

TMX – Tamoxifen

TNBC – Triple-negative breast cancer

TNFRSF6B – TNF Receptor Superfamily Member 6b

TNM - Tumor, Node and Metastases

TP53 - Tumor protein p53

TP53INP1 - Tumor Protein P53 Inducible Nuclear Protein 1

TP63 - Tumor Protein P63

TPM1 – Tropomyosin 1

TRBP - Trans-activation response RNA binding protein

TRBP - Trans-activation response RNA binding protein

tRNA - Transfer RNAs

UNKN – Unknown

UTR - untranslated region

VIM – Vimentin

WHO - World Health Organization

XBP1 - X-Box Binding Protein 1

XPO5 - Exportin-5

yr – Years

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

ZEB - Zinc finger E-box-binding homeobox

ZNF217 - Zinc finger protein 217

INTRODUCTION

Breast Cancer

Breast Cancer overview

Epidemiology and Risk Factors

Breast cancer (BrCa) is the second most common cancer worldwide and the most frequent cancer among women (1). In 2012, the estimated age-adjusted annual incidence of BrCa in 40 European countries was 92.8/100 000 and the mortality 23.1/100 000 (2). In Portugal, BrCa was the leading cancer in 2012 and the first cause of cancer death in women (**Figure 1**) (3). BrCa in males is rare, contributing to ~1% of cases (4).

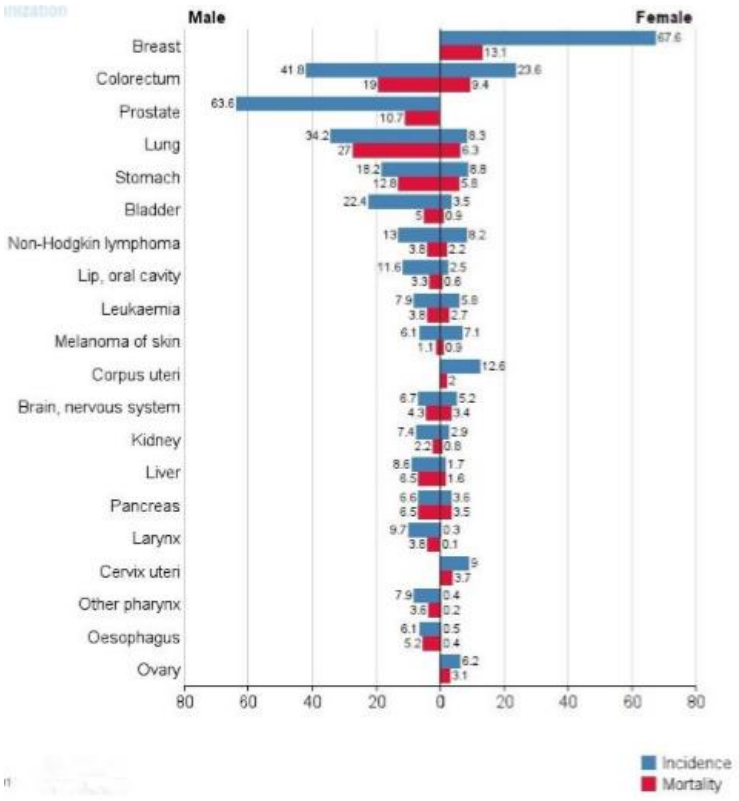

Figure 1. Estimated Age-Standardized Incidence and Mortality Rates (per 100 000) in Portugal in 2012. Adapted from (3).

BrCa incidence increased after the application of mammography screening and it is still increasing with the ageing of the population. On the contrary, due to earlier diagnosis and increased treatment with the implementation of adjuvant chemo-, radio- and endocrine-therapies (ET), the mortality rate has been decreasing in most Western countries, while

BrCa prevalence is increasing (3). Indeed, despite the high global incidence, BrCa is the fifth cause of death from cancer for both genders. However, it constitutes the leading cause of cancer-related deaths in European women and in women worldwide (5, 6). Ten-year survival of BrCa exceeds 70% in most European regions, being 89% for local and 62% for regional disease (7). Approximately 5% of patients present with distant metastasis at time of diagnosis and 10-15% of patients develop distant metastasis within the first 3 years (8). Importantly, metastatic BrCa is almost always incurable (9).

Multiple factors have been associated with an increased risk of developing BrCa with distinct scientific evidence (**Appendix I**); however, it has been estimated that approximately 50% of women who develop BrCa have no identifiable **risk factors** excepting increased age and female gender (10). Indeed, BrCa incidence rises sharply with age, with the highest rate of BrCa being observed among women aged 75 to 79 (11). In addition, BrCa incidence also differs by race and ethnicity (12, 13). For instance, in women less than 40 years, BrCa incidence is higher in African-american women than in Caucasian women; however, the contrary occurs among those aged 40 years or older (12).

Moreover, some benign breast lesions, e.g., proliferative disease without and with atypia, have been associated with a slight increase in the subsequent risk of developing invasive BrCa (14). Furthermore, women with a family history of BrCa, especially if the affected family member was diagnosed at a younger age, have an increased risk of developing BrCa (15). Mutations in the BrCa susceptibility genes *BRCA1, DNA repair associated* (*BRCA1*) and *BRCA2, DNA repair associated* (*BRCA2*) were also associated with a significant increase in the lifetime risk of BrCa, that ranges from 26 to 85% (10), however, these account for 5 to 10% of all BrCa, and are most strongly related to BrCa occurring in younger premenopausal women (16). Additional genes such as *Tumor Protein p53* (*TP53*), associated with the Li-Fraumeni syndrome (17) and *Phosphatase and Tensin homolog* (*PTEN*), associated with the Cowden syndrome (18), play a minor role in familial BrCa syndromes.

Many of the established BrCa risk factors can be attributed to some means of elevated estrogen exposure, as many studies have consistently demonstrated that increased levels of endogenous estrogen are associated with increased BrCa risk in postmenopausal women (19), which might be explained by estrogen's capacity to stimulate proliferation of both normal and malignant breast cells (20). Reproductive factors linked to an increase in

BrCa risk include early age at menarche and late age at menopause. On the other hand, parity and premenopausal oophorectomy have a protective effect on BrCa risk (21). Breastfeeding also appears to contribute to a reduced risk of BrCa, although parity may be a confounding factor (22). Besides reproductive factors, in postmenopausal women, obesity and hormone replacement therapy (HRT) are also associated with increased BrCa risk (23).

There is also a well-established relationship between exposure to ionizing radiation and the risk of developing BrCa, with the risk being inversely associated with age at radiation exposure (24).

Finally, there is also a substantial interest in whether dietary or lifestyle factors modify BrCa risk. For instance, vegetable consumption and physical activity seem to have a moderate protective effect (25), while high-fat diets (26) and alchohol consumption (27) seem to be associated with higher rates of BrCa.

Diagnosis and screening

The **diagnosis** of BrCa is initially based on clinical examination, which includes bimanual palpation of the breast and locoregional lymph nodes, in combination with imaging (28). The current *in vivo* diagnostic tools for the detection of early-stage BrCa are mammography, for which accuracy is greatly affected by age and consequently denser breasts (29) and in specific cases magnetic resonance imaging (MRI) (30). A number of circulating tumor markers [e.g., carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3)] are sometimes used in the management of BrCa, but due to their low sensitivity they are not for screening intent but only for disease monitoring (31). Indeed, the presence or absence of carcinoma can only be correctly determined by tissue sampling and pathological examination of the primary tumor and cytology/histology of the axillary nodes, if involvement is suspected (32). Thus, biopsy remains the standard technique for diagnosing both palpable and non-palpable breast abnormalities (32).

In order to detect BrCa at a pre-clinical stage, several countries have established population-based mammography **screening programs** (33). Mammography screening, every 2 years, has shown the greatest mortality reduction benefit in women between 50-69 years (32). In women with familial BrCa, annual MRI concomitantly or alternating every 6

months with mammography, starting 10 years younger than the youngest case in the family, is recommended (30).

Histological subtypes

BrCa is a highly heterogeneous disease with distinct biological features and clinical outcomes. Currently, the World Health Organization (WHO) identifies more than 20 histological types, using a classification scheme based on the growth pattern and cytological features of the tumor cells, and independent of the site of origin in the breast (34).

The majority of BrCa has origin at epithelial cells and can be subdivided into *in situ* and invasive carcinomas. ***In situ* carcinomas** might be lobular carcinoma *in situ* (LCIS) or ductal carcinoma *in situ* (DCIS), and are defined as pre-invasive lesions in which neoplastic epithelial cells proliferate confined to the ductal/lobular tree of the breast without evidence of invasion through the basement membrane (35). DCIS is more frequent than LCIS and, with the implementation of mammographic screening programs, represents about 20-25% of newly diagnoses BrCa (34, 35). **Invasive carcinomas** are the most common lesions, representing 70-80% of all BrCa malignant neoplasms (35). Invasive carcinomas can generally be grouped in two categories: invasive carcinoma of no special type (NST), also known as invasive ductal carcinoma (IDC), and special subtypes carcinomas (SSC), with IDC representing up to 75% of all invasive carcinomas (34). The most common lesion of the SSC group is the invasive lobular carcinoma (ILC), representing 5-15% of all BrCa. Tumors that have both SSC pattern (10-49%) and NST pattern are categorized as mixed (34).

Prognostic and predictive biomarkers

A prognostic factor is defined as a measurement taken at the time of diagnosis or surgery that is associated with the innate aggressiveness of untreated BrCa and thus outcome, while a predictive factor is a measurement that predicts response or lack of response to a specific treatment (10). In clinical practice, many biomarkers have both prognostic and predictive significance.

Histologic grade, determined by using the Nottingham combined histologic grade proposed by Elston and Ellis (**Appendix II**), has been shown to have prognostic significance and is a key component for clinical decision-making (34, 36). Moreover, the Tumor, Node and

Metastases (TNM) staging system published by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) is based on established clinical and pathological prognostic factors, namely tumor size (T), the extent of axillary lymph node involvement (N) and the spread of distant metastases (M) (34) (**Appendix III**). This staging system allows the establishment of five stages in order to evaluate the disease extension and the patient's prognosis (**Appendix IV**).

However, grade and stage display limited value as sole prognostic factors and other prognostic and therapy predictive biomarkers have been introduced in the daily practice (32). Indeed, **estrogen receptor** (ER), **progesterone receptor** (PR) and **human epidermal growth factor 2 receptor** (HER2) *status* assessed by immunohistochemistry are also evaluated in all breast tumors.

ER and **PR** are two nuclear transcription factors activated by the hormones estrogen and progesterone, respectively, and are the most important and useful predictive factors currently available. Indeed, ER expression is a strong ET predictive marker of response (34, 37). Besides, PR-negative patients have a higher relative risk of disease recurrence after ET in comparison to patients with PR-positive tumors. Moreover, ER-negative tumors have a poorer prognosis in the first years after diagnosis, but after 5 to 10 years ER-positive tumors have the poorer outcome (38). Importantly, BrCa may relapse in ER-positive patients more than 20 years after the diagnosis (39).

HER2 gene is an oncogene localized in the chromosome 17 and is amplified in approximately 15% of BrCa tumors yielding overexpression of its coding protein, a growth factor receptor present in breast epithelial cell surface (40). HER2 *status* has both prognostic and predictive significance. Although HER2-positive BrCa patients have a worse prognosis, HER2 positivity is predictive of a favorable response to HER2-targeted therapy (e.g. trastuzumab) (34, 37). In addition, HER2 positivity is also a response predictor of anthracycline- and taxane-based therapy (41) and of unresponsiveness to ETs (42).

Proliferation markers such as the **Ki-67** may supply additional useful prognostic information (43). Furthermore, patient **age** has been consistently shown to be a prognostic factor, as very young BrCa patients have a poorer prognosis than older patients (10).

Nonetheless, clinical decisions based upon one or small number of genes or their coding proteins in the tumor tissue have failed to predict patients' outcome, which prompt to the development of other prognostic assays, based in the examination of multiple expressed genes, e.g., Oncotype DX [21-gene derived recurrence score (RS)], MammaPrint (Amsterdam 70-gene derived RS) and PAM50 (50-gene derived RS) (44), for both classification and prognostication of individual tumors. However, widespread use of **gene-expression profiling** in clinical practice remains limited, primarily due to the high costs and technical difficulty encountered when carrying out high-throughput gene-expression profiling, in addition to the invasive diagnostic procedures, since tissue biopsies are required.

Molecular Subtypes

Based on gene expression profiling, BrCa is often classified into four well-established intrinsic subtypes (**Table 1**), which are associated with distinct biological features and clinical outcomes (45, 46). These intrinsic subtypes can be defined by gene expression profiling using multiparameter molecular tests such as the PAM-50 (47, 48). Luminal tumors typically express luminal cytokeratins (CK) 8 and 18 (44). Luminal tumors can be further subdivided into **Luminal A** and **Luminal B**. The major molecular distinctions between luminal A and B tumors are based in the ER-related genes' higher expression in luminal A tumors, whereas luminal B tumors exhibit a higher expression of proliferation-related genes (49). ER-negative tumors encompass two subtypes: the **HER2-enriched subtype**, characterized by high expression of several genes in the HER2 amplicon at 17q22.24, including HER2 (48) and the **basal-like** subtype, characterized by CK 5/6 and CK17 and basal epithelial genes' expression (48).

However, due to financial constraints, surrogate approaches have been developed for routine clinical practice using more widely available immunohistochemistry (IHC) assays for ER, PR and Ki-67 index, together with IHC and/or *in situ* hybridization for HER overexpression/amplification (32). Luminal A tumors do not overexpress HER2 and have a low Ki-67 index, while luminal B tumors can be HER2 negative or positive. Commonly, Luminal B negative for HER2 expression has either high Ki-67 value or a negative or low PR expression. Eighty % of Basal-like tumors overlap with triple-negative tumors, negative for both hormone-receptors and HER2 expression (50) (**Table 1**).

These subtypes contribute to insights into cancer initiation and progression and might be of value in assessing prognosis and prediction response to therapy, guiding clinical management (32, 51, 52).

Table 1. Breast Cancer molecular subtypes characterization (1, 30, 32, 48, 49, 53-57).

Breast cancer subtypes		**Clinicopathological surrogate markers**	**Incidence (%)**	**Signature genes**	**Prognosis [5/10-year survival rate (%)]**	**Adjuvant therapeutic options**
Luminal A		ER^+ PR high * $HER2^-$ Ki-67 low **	50-60	*ESR1* and/or *PGR*, *KRT8*/18, *GATA3*, *XBP1*, *FOXA1* and *ADH1B*	Show the most favorable clinical features in the first years of follow-up [95/85]	ET alone in the majority of cases + Cht if high tumor burden (≥N3, ≥T3)
Luminal B	**$HER2^-$**	ER^+ $HER2^-$ Ki-67 high or PR low	10-20	*ESR1* and/or *PGR*, *KRT*8/18, *FGFR1*, *ERBB1*, *MKI67 and*/or *CCNE1*, *CCNB1* and *MYBL2*	Show less favorable clinical outcomes compared with luminal A tumors [80/75]	ET + Cht for the majority of cases
	$HER2^+$	ER^+ $HER2^+$ Any Ki-67 Any PR				ChT + anti-HER2 + ET for all patients
Basal-like		ER^- PR^- $HER2^-$	10-20	*KRT*5/6, *KRT*17, *ERBB1* and/or *KIT*, FOXC1, TP63, *CDH3*, VIM and *LAM*	Poor clinical features and survival [75/70]	ChT
HER2-enriched		$HER2^+$ ER^- PR^-	10-15	*ERBB2* and *GRB7*	Poor clinical features and survival [65/60]	ChT + anti-HER2

* Suggested cut-off value is 20% (32) ** Ki-67 scores should be interpreted in the light of local laboratory median values (32)

Abbreviations: ER – estrogen receptor; PR – progesterone receptor; HER2 - Human Epidermal growth factor Receptor 2; *ESR1* - Estrogen Receptor 1; *PGR* - Progesterone Receptor; *KRT* – keratin; ; *GATA3* - GATA Binding Protein 3; *XBP1* - X-Box Binding Protein 1; *FOX* - Forkhead Box; *ADH1B* - Alcohol Dehydrogenase 1B (Class I), Beta Polypeptide; *FGFR1* - Fibroblast Growth Factor Receptor 1; *ERBB* - Erb-B2 Receptor Tyrosine Kinase; *MKI67* - Marker Of Proliferation Ki-67; *CCN* – Cyclin; MYBL2 - MYB Proto-Oncogene Like 2; *MYBL2* - MYB Proto-Oncogene Like 2; *KIT* - KIT Proto-Oncogene Receptor Tyrosine Kinase; *TP63* - Tumor Protein P63; *CDH* – Cadherin; *VIM* – vimentin; *LAM* – laminin; *GRB7* - Growth Factor Receptor Bound Protein 7; Cht – chemotherapy.

Therapeutic approaches

Currently, several treatment strategies available for BrCa patients are based on the tumor burden/localization and biology, as well as age, general health status and patient's preferences (58). Indeed, BrCa is the pioneer of personalized medicine in oncology.

Neoadjuvant treatment might be performed in multifocal disease, or in order to downsize locally advanced and large unifocal unresectable primary tumors that would require mastectomy (58). All modalities that will be described for adjuvant systemic treatment might also be used as neoadjuvant therapy.

Regarding **local treatment**, breast-conserving surgery is amenable in the vast majority of newly diagnosed cancers, while mastectomy is carried out for larger tumor sizes, tumor multicentricity, inability to achieve negative surgical margins after multiple resections, prior radiation to the chest wall/breast or other contraindications to radiotherapy (RT) or even when patient demands (59). The treatment of regional lymph nodes can be performed using two approaches: axillary clearance and sentinel lymph node biopsy (SLNB). Presently, SLNB is the standard care for axillary staging in early, clinically-negative BrCa, due to the associated reduced morbidity (60). Conventional axillary lymph node clearance is mandatory in the presence of macrometastatic spread in the sentinel node (61). After breast-conserving surgery and after mastectomy in node-positive patients RT is highly recommended (62, 63).

Adjuvant systemic therapy, to prevent BrCa recurrence by eradicating micrometastatic tumor deposits present at diagnosis, comprises three modalities: chemotherapy, anti-HER2 therapy (trastuzumab) and ET.

Most luminal A tumors, except those with the highest risk of relapse, do not require adjuvant chemotherapy, whereas most luminal B tumors, especially those with HER2 overexpression, benefit from this modality (64). Triple-negative tumors and HER2-overexpressing tumors benefit from this systemic therapy. HER2-positive patients are also treated with a monoclonal antibody that interferes with HER2 (e.g. trastuzumab) (64).

ET, which blocks ER activation, is indicated for all ER-positive patients to stop or slow the growth of hormone-sensitive BrCa (58). ETs include: selective ER modulators (SERMS), such as tamoxifen, which competes with the estrogen hormone estradiol (E_2) for binding ER and inhibit ER transcriptional activity in BrCa cells by recruiting corepressors; selective

SER down-regulators (SERDs), such as fulvestrant, that in addition to binding to ER also stimulate its degradation; and aromatase inhibitors (AIs), such as letrozole (non-steroidal/reversible inhibitor) and exemestane (steroidal/reversible inhibitor) that suppress estrogen production in adipose tissue and other peripheral tissues by blocking the activity of the aromatase enzyme that synthesis estrogen via aromatization of androgens. Ovarian suppression with the use of gonadotropin-releasing hormone (GnRH) agonists or ovarian ablation remains controversial (65), and GnRH agonists should only be used as an alternative to cyclophosphamide/methotrexate/fluorouracil-type chemotherapy when this treatment is not tolerated (66) or in patients with contraindications to tamoxifen (32). The choice of the agent is primarily determined by patients' menopausal status, being AI therapy recommended for postmenopausal women, while in premenopausal women tamoxifen (5-10 years) is the standard ET (67, 68).

Although ET results in substantial improvement of patients' outcome, treatment resistance has become a major limitation (49), affecting 30-40% of ER-positive BrCa patients, with all those treated in the metastatic setting eventually progressing (69). Among the luminal subtype, luminal A tumors display the best clinical outcomes after ET.

Endocrine Resistance

According to 3rd ESO–ESMO International Consensus Guidelines, endocrine resistance may be defined as primary endocrine resistance when patients relapse within the first 2 years of adjuvant ET, or as secondary (acquired) endocrine resistance, when patients relapse while on adjuvant ET after the first 2 years of treatment or within the 12 months after completing treatment (70). Detailed information on the biology of the several molecules implicated in endocrine resistance *in vitro* and the means by which they cause resistance is summarized in several recent reviews (71-75).

As mentioned before, ER expression is currently the most important ET response biomarker and altered ER expression contributes to the development of endocrine resistance (76). However, ER has been found to be absent in only 15-20% of endocrine-resistant cancers, implicating other mechanisms in the development of endocrine-resistance (77).

ER may have a genomic or a non-genomic signaling. **Genomic ER signaling** includes the classic mode of estrogen signaling, in which E_2 binds to ER inducing a conformation

change that leads to its activation and binding to DNA sequences denominated estrogen response elements (ERE). Alternatively, the E_2-ER complex may interact with other transcription factors to facilitate their binding to serum response elements (SREs) and the activation of transcription (78). Ligand-independent ER activation has been shown to play a role in endocrine resistance, and may be caused, for example, by mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) phosphorylation (79). Furthermore, increased ER splice variants' expression, e.g., ERα36, has also been linked to endocrine resistance (80, 81).

A small percentage of ER is localized at the plasma membrane and E_2 binding to these receptors mediates a **non-genomic ER signaling** by initiating rapid intracellular phosphorylation cascades' activation mediated by phosphoinositide3-kinase (PI3K) or Akt (82, 83). Indeed, several mechanisms of endocrine resistance include amplification or abnormal activation of multiple growth factor signaling pathways (84), including HER2 (85), MAPK (86) and PI3K (87). In some cases, deregulation of these signaling pathways occurs as a result of genetic or epigenetic modifications (88), whereas in other cases, deregulation reflects aberrations in upstream regulators, such as the activation of Akt in association with the loss of PTEN expression (89, 90).

In addition to the deregulation of estrogen signaling, unrelated mechanisms that endows tumor cells with alternative proliferative and survival stimuli, e.g. disturbances in the apoptosis pathways, might also play a significant role in endocrine resistance (91). Besides, a link between epithelial-to-mesenchymal transition (EMT) and endocrine resistance has already been reported in BrCa (92, 93).

Epigenetics

The concept of epigenetic has been evolving through the years, and currently is defined as the study of mechanisms that initiate and maintain patterns of gene function and regulation without affecting the nucleotide sequence, in a heritable manner (94). The potential role of epigenetic processes to complement genetic changes in cancer has been already accepted. Indeed, epigenetic aberrations arise early in the carcinogenesis process and are therefore potential targets for early detection (94). In addition, epigenetic alterations may be reversed by drugs, providing the opportunity to design epigenetic therapies (95). Four different mechanisms of gene expression regulation are comprised in the field of

epigenetics, namely: DNA methylation, covalent histone modifications, histone variants and non-coding RNAs (ncRNAs) (94).

DNA methylation

DNA methylation commonly occurs with the addition of a methyl group at the 5' position of a cytosine ring inside CpG dinucleotides (96, 97). CpG sites are concentrated at 5'-gene regulatory regions, denominated CpG islands, which are characterized by having 200 or plus base pairs (bp), CG content of at least 50% and a ratio of observed/expected CpG frequency of at least 0.6 (98, 99). About 60% of human gene promoters contain a CpG island, which are usually unmethylated in normal cells (98, 100). DNA methylation is performed by specific enzymes denominated DNA methyltransferases (DNMTs), either by *de novo* (DNMT3A and DNMT3B) or for the maintenance of methylation (DNMT1) (98, 99). This epigenetic alteration may be removed through demethylation, which can take place as a passive process due to the impairment of DNA methylation maintenance or as an active mechanism that remains controversial (101, 102).

DNA methylation is responsible for gene expression inhibition, directly by avoiding the binding of transcription factors and/or indirectly by recruiting methyl-CpG binding domain (MBC) proteins, which in turn promote the recruitment of histone modifying and chromatin-remodeling complexes that lead to repressive states of chromatin organization (97, 98).

Several studies throughout the last two decades have been showing the DNA methylation deregulation in cancer (103). Indeed, tumor cells are characterized by a global loss of DNA methylation and also by hypermethylation at specific CpG islands, especially in promoter regions of tumor-suppressor genes (95, 98). In BrCa, several genes have been identified as being hypermethylated, including genes coding metabolic enzymes [e.g. Glutathione S-Transferase Pi 1 (*GSTP1*)] and cell cycle inhibitor genes [e.g. Ras Association Domain Family Member 1 (*RASSF1A*)] (104).

Covalent histone modifications and histone variants

The basic units of chromatin are nucleosomes, core particles that consist of about 147 bp of DNA wrapped around an octamer consisting of two copies of each core histones - H2A, H2B, H3 and H4 - and a molecule of histone H1 that seals the two turns of DNA (105). Chromatin is not a static construction, but varies between two different states: heterochromatin, a highly packaged conformation that commonly contains inactivate

genes; and euchromatin, a more relaxed form associated with an active transcription (106).

Histone proteins present an unstructured N-terminal tail that may undergo **post-translation modifications**, which affect the chromatin structure (105, 107, 108). The most well-known modifications are histone acetylation and histone methylation. Histone acetylation is performed by a family of enzymes denominated histone acetyltransferases (HATs), and histone deacetylation is performed by histone deacetylases (HDACs) (108). Acetylation occurs in lysines and leads to the promotion of gene expression (105). Histone methylation, in turn, can occur in lysine (mono-, di- or trimethylation) or arginine (mono- or di-methylation) residues, leading either to transcription repressing or gene activation (105). Histone methyltransferases (HMTs), responsible for the addition of methyl groups to residues, and histone demethylases (HDMs) that can reverse such change, are the two families of enzymes that control histone methylation. The various histone modifications are interpreted by other proteins termed “readers”, which modify local chromatin structure to either stimulate or repress gene expression (108).

Post-translation modifications of histone tails have also been found deregulated in several cancers, including BrCa (109). For instance, *Enhancer of zeste 2* (EZH2), a methyltransferase responsible for the H3K27me3 mark, is found overexpressed in BrCa and correlates with poorer prognosis of BrCa patients (110).

Histone variants are non-allelic variants of the major histones that are key players in shaping chromatin structure and, when deregulated, may contribute to cancer initiation and progression (111). For instance, a direct role for H2A.Z in cancer was found in hormone-dependent breast, where an elevated level of H2A.Z expression significantly associated with metastasis to lymph node and with shorts overall survival (OS) (111-113)

Non-coding RNAs

The most-well studied sequences in the human genome are those of protein-coding genes, which comprises only 2% of the human genome. However, about 90% of the human genome is transcribed into RNA molecules (114). In the last years, it has become increasingly apparent that the non-protein-coding portion of the genome is of crucial importance, and particular emphasis has been given to ncRNAs. NcRNAs are composed of different classes depending on their size, interactions and activity, including some well-

known RNAs such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and small nucleolar RNAs (snoRNAs), as well as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (31).

As expected by their functional importance within cellular context, ncRNAs are deregulated in human diseases, including cancer (114, 115). Indeed, new potential diagnostic, prognostic and predictive biomarkers, as well as therapeutic targets, have been emerging since the established role of ncRNAs in disease onset and development (116). MiRNAs involvement in cancer has been the most studied throughout the last years (114).

MicroRNAs

MiRNAs are a class of small [~22 nucleotides (nt)] non-coding single-stranded RNAs first discovered in *Caenorhabditis elegans*, in 1993 (117). MiRNAs have important roles in a wide range of biological processes at posttranscriptional level (118). Indeed, computational predictions estimate that each miRNA regulates hundreds of different messenger RNAs (mRNAs) and about 50% of the human transcriptome is subject to miRNA regulation (119).

Biogenesis and mode of action

MiRNAs genes are found dispersed across the genome either as single genes or in gene clusters that give rise to polycistronic transcripts from which the individual miRNAs are processed. MiRNAs genes may be located in intergenic regions or in transcription units in either sense or antisense orientation (**Figure 2**) (120). MiRNAs localized within introns have been denominated “mirtrons” (121). Besides, miRNAs may be transcribed from their own promotor, or be co-transcribed with the host gene in which they reside (120)..

MiRNAs pass through a long maturation process that begins in the nucleus and ends in the cytoplasm (**Figure 2**). Briefly, in the standard (canonical) miRNA biogenesis pathway, miRNAs genes are transcribed by RNA polymerase II, generating a large miRNA precursor (primary-miRNA) which contains one or more stem-loops or hairpin structures that are recognized and cleaved by the nuclear microprocessor complex consisting of the endonuclease Drosha and the Di-George syndrome critical region gene 8 (*DGCR8*) protein, originating an RNA hairpin intermediate (pre-miRNA) with two nt 3’ overhang (122). In turn, mirtrons seem to follow an alternative biogenesis pathway, being processed by the splicing machinery and originating debranched introns that mimic the structural features of precursor miRNAs without Drosha-mediated cleavage (123). From this point

on, canonical and alternative mechanisms merge following a common pathway. The pre-miRNA is actively transported from the nucleus to the cytoplasm through the Exportin-5 (XPO5), where it is cleaved by the endonuclease Dicer together with the trans-activation response RNA binding protein (TRBP), yielding a transitory miRNA duplex (118). Supported by the HSC70-HSP90 chaperone machinery, this duplex is loaded into an Argonaute (AGO) protein as a dsRNA and subsequent maturation steps expel one of the miRNA duplex strands, producing a mature RNA-induced silencing complex (RISC) (124).

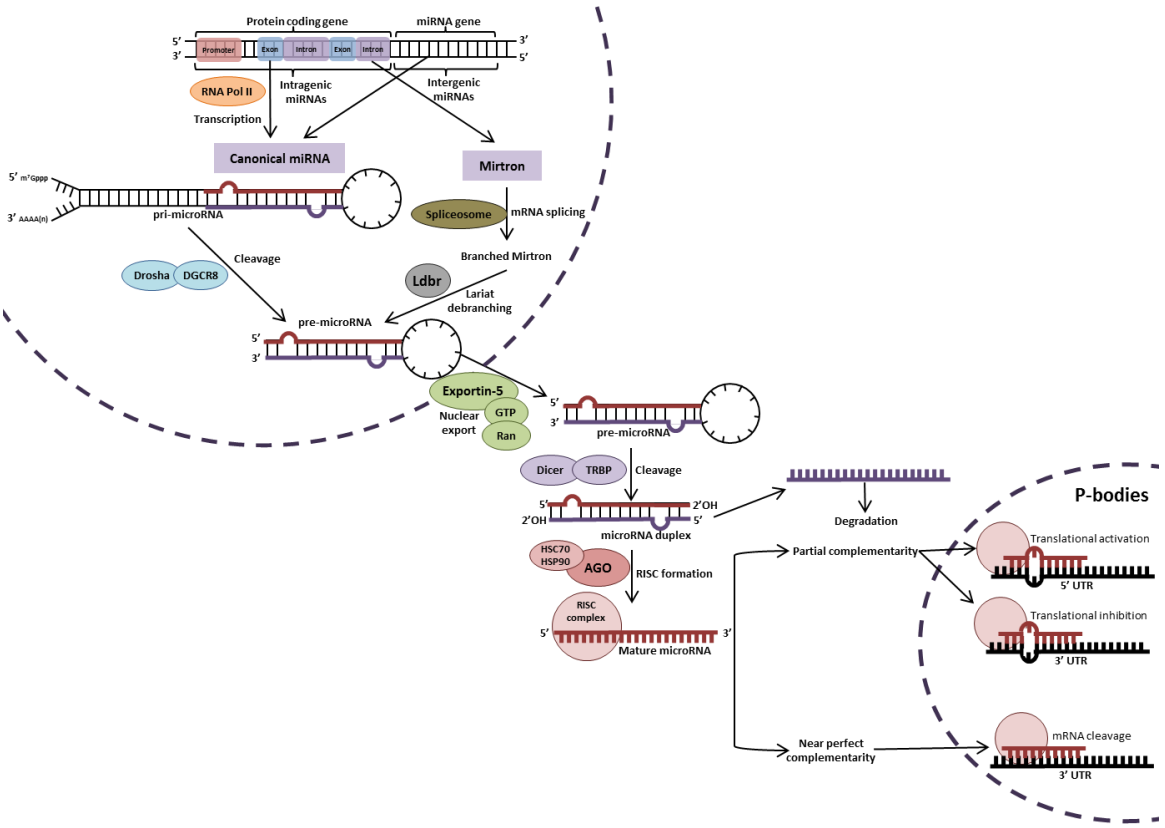

Figure 2. MiRNA biogenesis pathway: canonical and alternative pathway, from nucleus to cytoplasm. **Abbreviations:** miRNA – microRNA; POL – polymerase; DGCR8 - Di-George syndrome critical region gene 8; Ldbr - lariat debranching enzyme; GTP - Guanosine-5'-triphosphate; TRBP - Trans-activation response RNA binding protein; HSP - Heat shock proteins; UTR - untranslated region; RISC - RNA-induced silencing complex; AGO – Argonaute; Ran - RAs-related Nuclear protein. Amorim, Maria unpublished.

MiRNAs targets are identified through base-paring interactions between a 6-8 nt domain at the 5' end of the loaded miRNA, denominated "seed sequence", and an mRNA target [generally the 3' untranslated regions (UTR)]. MiRNAs can downregulate gene expression by either two posttranscriptional mechanisms: **mRNA cleavage** when the mRNA has perfect complementarity to the miRNA, or **translational repression**, when the

complementary between the miRNA and the mRNA is only partial (119). Initially, miRNAs binding sites were thought to be exclusively located in the 3'UTR of target mRNAs. Recently, 5'-UTR and open reading frames (ORF) were reported as containing target sequences for miRNAs function, increasing the complexity degree in miRNAs' research filed (125, 126). Indeed, miRNAs binding to the 5'UTR seems to upregulate the target mRNA translation (127). Recently, the so-called P-bodies were singled out as sites where translational repression occurs. Target mRNAs were found to be sequestered in the cytoplasmatic P-bodies away from the ribosomes thereby precluding their translation (128).

MicroRNAs deregulation in cancer

MiRNAs are known to play a significant role in cellular transformation and carcinogenesis, and several studies have been showing a differential miRNA expression profile and a global miRNA downregulation in human malignancies as compared with normal (129). Many changes occur in cancer cells that might influence, in a direct or indirect manner, miRNA expression, e.g. genomic rearrangements and abnormalities in miRNA processing genes or proteins (130, 131). Besides, recent studies have suggested that disrupted epigenetic alterations may also be involved in the dysregulation of miRNAs, particularly abnormal DNA methylation of CpG islands in the miRNAs promoter regions (131-133). Indeed, a comprehensive bioinformatics analysis found that about 50% of miRNA genes are associated with CpG islands, suggesting that several miRNAs might be candidate targets of the DNA methylation machinery (14).

Figure 3 exemplifies some of the miRNAs that are associated with the carcinogenesis process, and some of the target genes through which they exert their regulatory function (129). Indeed, the aberrant miRNA expression in human tumors is not just a casual association, but they can exert a causal role, as oncogenes (oncomiRs) or tumor suppressors, in different steps of the tumorigenic process. **OncomiRs** act by repressing the expression of tumor suppressor genes and are frequently upregulated in cancer. **Tumor suppressor** miRNAs act by targeting oncogenes and are frequently downregulated in cancer (129). However, this miRNA categorization may be inaccurate, as many studies have shown that miRNAs may present a dual function, with oncogenic or tumor suppressive properties based on tumor type and cellular context (134).

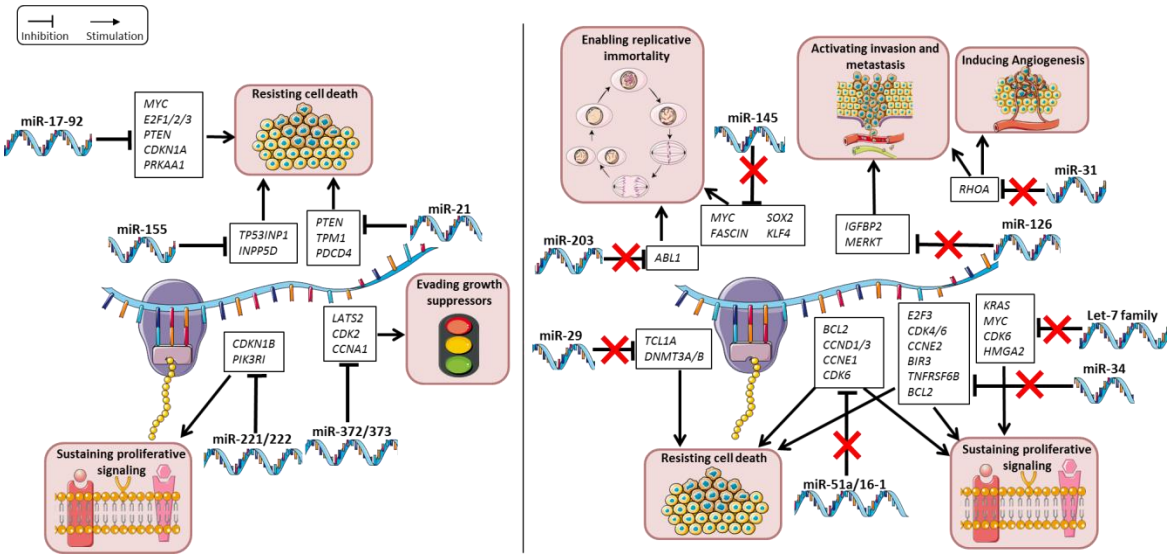

Figure 3. Examples of oncomiRs and tumor suppressor miRs associated with the carcinogenesis process, and some of the target genes through which they exert their regulatory function. **Abbreviations:** MYC – MYC proto-oncogene, bHLH transcription factor; E2F – Transcription factor E2F/dimerisation partner family protein; PTEN – Phosphatase and tensin homolog; CDKN – Cyclin-dependent kinase inhibitor PRKAA1 – Protein kinase AMP-activated catalytic subunit alpha 1; TP53INP1 - Tumor Protein P53 Inducible Nuclear Protein 1; INPP5D – Inositol polyphosphate-5-phosphatase D; TPM1 – Tropomyosin 1; PDCD4 – Programmed cell death 4; CDKN1B – Cyclin dependent kinase inhibitor 1B; PI3KR1 – Phosphoinositide-3-Kinase Regulatory Subunit 1; LATS2 – Large tumor suppressor kinase 2; CDK – Cyclin-dependent kinase; CCNA1 - Cyclin A1; ABL1 – ABL proto-oncogene 1, non-receptor tyrosine kinase; SOX2 – SRY-box 2; KLF4 – Kruppel like factor 4; TCL1A – T-cell leukemia/lymphoma 1A; DNMT – DNA methyltransferase; IGFBP2 – insulin like growth factor binding protein 2; MERKT – MER proto-oncogene, tyrosine kinase; RHOA – ras homolog family member A; BCL2 – BCL2, apoptosis regulator; CCN – cyclin; E2F3 – E2F transcription factor 3; TNFRSF6B – TNF Receptor Superfamily Member 6b; KRAS – KRAS proto-oncogene, GTPase; HMGA2 – high mobility group AT-hook 2; Amorim, Maria unpublished.

MicroRNAs and their use in the clinic

In recent years, miRNAs have been implicated in the clinical management of cancers at every stage (135). Firstly, miRNA expression profiles can be used as a **diagnostic** tool, since each tumor type seems to have a distinct miRNA signature that distinguishes it from normal tissues and other cancer type (136-138). Secondly, **prognostic** miRNA expression signatures may be identified within tumor groups (139-141). Thirdly, miRNAs may also function as **predictive** biomarkers, helping clinicians in the choice of the correct individual therapeutic approach (142). Lastly, miRNAs may be targets of cancer **therapeutic** intervention, either by inducing or re-expressing tumor suppressor miRNAs, or by downregulating oncomiRs (143).

In addition to offering an attractive option as stable biomarkers in the tumor tissues [e.g. formalin-fixed paraffin-embedded (FFPE) blocks and fresh frozen tissues], the use of circulating miRNAs as biomarkers is also possible, since tumor cells can release miRNAs stabilized by their incorporation into microvesicles or associated with protein complexes , which have shown stability in several bodily fluids (e.g. plasma, serum and urine) (144).

MicroRNAs and Breast Cancer

Amorim, Maria, et al. "Decoding the usefulness of non-coding RNAs as breast cancer markers." Journal of translational medicine 14.1 (2016): 265 (**Appendix V**).

MiRNAs and endocrine resistance

In recent years, several studies have been linking miRNAs deregulation with endocrine resistance (145-148). Some of the miRNAs identified are depicted in **Figure 4** along with their putative targets and functional implications**.**

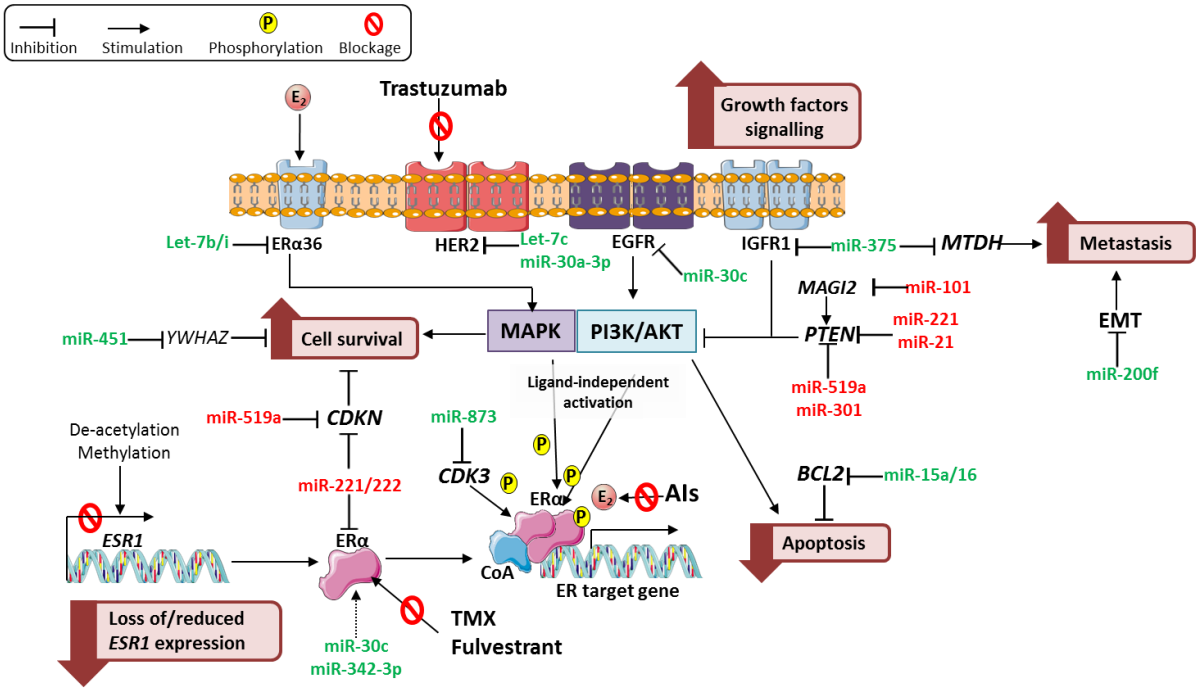

Figure 4. MiRNAs and their established targets involved in endocrine resistance. The miRNAs and their targets involved in several mechanisms associated with endocrine resistance, along with their functional implication (in pink boxes), including loss of/reduced *ESR1* expression, alternative growth factors signaling, including PI3K/Akt and MAPK signaling pathways, dysregulation of cell survival and apoptosis pathways, and increased metastasis. MiRNAs that confer sensitivity and resistance to endocrine therapies are depicted in gree and red, respectively. **Abbreviations:** ER – estrogen receptor; HER2 - Human Epidermal growth factor

Receptor 2; EGFR - epidermal growth factor receptor; IGFR1 - insulin-like growth factor 1 receptor; YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta; MTDH – metadherin; MAGI2 - membrane-associated guanylate kinase inverted 2; PTEN - Phosphatase and tensin homolog; EMT - epithelial–mesenchymal transition; CDKN - Cyclin-dependent kinase inhibitor; CDK3 - cyclin dependent kinase 3; BCL2 - BCL2, apoptosis regulator; PI3K/AKT - phosphoinositide 3-kinase/Protein kinase B; ESR1 - estrogen receptor 1; TMX – Tamoxifen; AIs – aromatase inhibitors; E_2 – Estradiol; miR – microRNA; miR-200f – miR-200 family. Amorim, Maria unpublished.

For instance, whereas decreased ER expression and endocrine resistance may be due to miR-221/222 overexpression (149, 150), miR-342-3p expression is positively correlated with ER mRNA transcript levels, being downregulated in tamoxifen refractory BrCa (151). MiRNAs have also been implicated in altering post-translational ER modifications. For instance, miR-873 directly targets the *cyclin dependent kinase 3* (*CDK3*) transcript that phosphorylates ER inducing its ligand independent activation, and the downregulation of miR-873 has been identified in endocrine resistant BrCa cell lines (152).

Furthermore, miRNAs regulating BrCa cells' growth, survival and apoptosis may also be implicated in loss of responsiveness to ET by endowing tumor cells with alternative proliferative and survival stimuli (91). Indeed, miR-519a associated with worse prognosis In luminal BrCa patients, directly targeting the transcripts of *cyclin dependent kinase inhibitor 1A* (*CDKN1A*) and *PTEN*, allowing for enhanced signaling of the *PI3K* growth and survival pathway (153).

Furthermore, miRNA-mediated endocrine resistance might be related with EMT and BrCa cells metastatic potential, as miR-200 family (miR-200f) members were found downregulated in endocrine-resistant BrCa *vs.* endocrine-sensitive cell lines, acting as major regulators of EMT (154, 155).

Table 2 summarizes some of the miRNAs involved in endocrine resistance/sensitivity, along with their putative targets and biological samples in which they were studied.

Table 2. Non-coding RNAs involved in response (sensitivity/resistance) to endocrine therapies along with their putative targets/mechanism.

ET	Role	miRNA	Putative target	Agent	Samples	Refs.
AntiE	S	**miR-375**	*MTDH*	TMX	Cell lines	(156)
		miR-873	*CDK3*			(152)
		miR-320a	*ARPP19*, *ESRRG*			(157)
		Let-7b/i	*ESR1* (ER-α36 variant)			(158)
		miR-451	*YWHAZ*			(159)
		miR-17/20	*CCND1*			(160)
		miR-148a miR-152	*ALCAM*			(161)
		miR-200c/b	*ZEB1*/2	TMX and FULV		(155)
		miR-15a/16	*BCL2*	TMX	Cell lines and xenografts	(162)
		miR-342-3p	*BMP7*, *GEMIN4*		Cell lines and tumor tissues	(151)
		miR-26a	*EZH2*		Tumor tissues	(163)
		miR-30c	*EGFR*			(146)
		miR-10a miR-126	-			(164)
	R	**miR-10b**	*HDAC4*	TMX	Cell lines	(165)
		miR-519a	*CDKN1A*, *PTEN*, *RB1*		Tumor tissues and cell lines	(153)
		miR-221/222	*ESR1*, *CDKN1B*, *CTNNB1*	TMX and FULV		(166-168)
		miR-301	*FOXF2*, *PTEN*, *BBC3iso2*, *COL2A1*	TMX	Tumor tissue, cell lines and xenografts	(169)
		miR-155	*SOCS6*			(170)
		miR-210	*EFNA3*, *E2F3*, *RAD52*, *FGFRL1*, *MET*		Tumor tissue	(147)
AIs	S	**Let-7f**	*CYP19A1*	LET	Cell lines	(171)
		miR-125b let-7c	*ERBB2*	LET and ANA	Tumor tissues and cell lines	(172)
	R	**miR-128a**	*TGFBR1*	LET	Cell lines	(173)

Abbreviations: miR – microRNA; lncRNA – long non-coding RNA; S – sensitivity; R – resistance; ET – endocrine therapies; AntiE – anti estrogen; AIs – aromatase inhibitors; ANA – anastrozole; FULV – fulvestrant; DSCAM-AS1 - DSCAM Antisense RNA 1; BCAR4 - breast cancer anti-estrogen resistance 4; MTDH - Metadherin; CDK - cyclin-dependent kinase; ; ARPP19 - cAMP-regulated phosphoprotein 19; ESRRG - estrogen related receptor gamma; YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta; CCND1 - cyclin D1; ALCAM - Activated Leukocyte Cell Adhesion Molecule; ZEB - zinc finger E-box-binding homeobox; BCL-2 - B-cell lymphoma 2; BMP7 - bone morphogenetic protein 7; GEMIN4 - gem (nuclear organelle)-associated protein 4; EZH2 - Enhancer of zeste homolog 2; EGFR - epidermal growth factor receptor; HDAC4 - Histone deacetylase 4; CDKN - Cyclin-Dependent Kinase Inhibitor ; PTEN - phosphatase and tensin homolog; RB1 – retinoblastoma 1; ESR1 - Estrogen Receptor 1; CTNNB1 - Catenin Beta 1; FOXF2 - forkhead box F2; BBC3iso-2 - BCL2 Binding Component 3 isoform 2; COL2A1 - collagen type II alpha 1; SOCS - suppressor of cytokine signaling; EFNA3 - Ephrin A3; E2F3 - E2F transcription factor 3; RAD52 - RAD52 homolog, DNA repair protein; FGFRL1 - fibroblast growth factor receptor-like 1; MET - MET Proto-Oncogene, Receptor Tyrosine Kinase; CYP19A1 - cytochrome P450 family 19 subfamily A member 1; ERBB2 - erb-b2 receptor tyrosine kinase 2; TGFBR1 - transforming growth factor, beta-receptor 1; BCL2L11 - BCL2 like 11; ZNF217 - zinc finger protein 217.

AIMS

The overall hypothesis of this Master Dissertation is that miRNAs that are deregulated in endocrine-resistant disease may be biologically and clinically relevant for luminal BrCa patients submitted to adjuvant ET. Thus, the main goal of this master dissertation is to identify miRNAs able to predict endocrine resistance among luminal BrCa patients undergoing ET, through the comparison of expression levels between BrCa cases that did or did not develop endocrine-resistance in long term follow-up. This might enable stratification of luminal BrCa cases into a low-risk subgroup, for whom additional adjuvant systemic treatment can be safely omitted, and patients who are at high-risk for recurrence potentially allowing the detection of resistance to ET at an early stage. Furthermore, we also intend to evaluate miRNA expression in normal breast and non-luminal tumor tissues to characterize the "baseline" *status* of miRNAs expression in healthy women and to evaluate miRNAs potential as diagnostic biomarkers.

Thus, the specific aims of this work were:

- Identifying a panel of deregulated miRNAs in endocrine-resistant tumors by a global expression assay in a discovery cohort composed of luminal A and B breast tumors from patients treated with adjuvant ET and with different outcomes;
- Validate some of the identified miRNAs in a larger set of tissues samples, including luminal tumor tissues to evaluate their predictive and prognostic potential, as well as non-luminal tumor tissues and normal breast tissues to evaluate their diagnostic potential;
- Evaluate the expression of the validated miRNAs in a cohort of primary tumor tissues and the paired metastasis in order to analyze their expression after the development of the resistance, rather than just before the therapy.

MATERIAL AND METHODS

Patients and samples collection

For this project, 176 BrCa samples were prospectively collected, after informed consent, from patients without metastasis at diagnosis aged between 40 and 75 years, which were submitted to surgery as first treatment from 1995 to 2002 at the Portuguese Oncology Institute of Porto (IPO Porto). Of these, 136 BrCa were luminal BrCa treated with adjuvant ET (with or without other adjuvant modalities). The remaining 40 BrCa samples were non-luminal disease (12 HER2-enriched and 28 triple-negative breast tumors). Furthermore, 26 normal breast tissue samples were collected from reduction mammoplasties of contralateral breast of BrCa patients. All specimens belonged to patients without BrCa hereditary syndrome and showed no evidence of preneoplastic/neoplastic lesions. After surgical resection, samples were immediately frozen at -80°C. Relevant clinical data was retrieved from patients' charts. Additionally, 5 μm frozen sections were cut and stained with hematoxylin-eosin (H&E) staining for BrCa confirmation, ensuring that samples contained at least 70% of tumor cells, and that tissues obtained from reduction mammoplasties harbored normal epithelial cells. This study was approved by institutional ethical committee (CES 120/015).

Luminal BrCa patients submitted to surgery as first treatment who did adjuvant ET (with or without other adjuvant modalities) and from whom both primary and metastatic tumors were available as FFPE tissue blocks were also included in this study. All primary tumors were invasive carcinomas and all samples had been formalin-fixed using the same standard procedure. Four μm sections were cut from each tissue block and stained with H&E, followed by a pathologist examination to select the most representative tumor lesion. The blocks containing the highest content of tumor tissues were then selected. All cases were revised by an experienced pathologist and graded according to Bloom and Richardson's Modified system and staged according to the AJCC system (34).

Breast cancer subtyping

IHC was performed to identify the molecular subtype of each tumor tissue included in this study. Commercially available antibodies were used for ER (Clone 6F11, mouse, Leica), PR (Clone 16, mouse, Leica), HER2 (Clone 4B5, rabbit, Roche) and Ki-67 (Clone MIB-1, mouse, Dako). IHC was carried out in BenchMark ULTRA (Ventana, Roche) using ultraView Universal DAB Detection Kit (Ventana, Roche) according to manufacturer's

instructions. Each case was evaluated by an experienced pathologist and was classified according to the College of American Pathologists recommendations (174). For HER2, cases with score 2+ were further assessed by fluorescence in situ hybridization. Each case was categorized according to ESMO guidelines (32). Cutoffs for Ki-67 and PR expression were 15% and 25% of positive cells, respectively.

MicroRNA expression analysis

RNA extraction from fresh frozen tissues

Total RNA was extracted from fresh frozen tissues using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. Briefly, 12 μm sections from fresh frozen tissues were cut and placed in 2mL tubes. One mL of TRIzol® Reagent was added to the tissue-containing tubes followed by homogenization. Following an incubation of 5 minutes at room temperature, 200 μL of chloroform (Merck, Darmstadt, Germany) were added. This mixture was vortexed, incubated for 3 minutes at room temperature, and again centrifuged for 15 minutes at 10600 rpm and 4°C. Then, the upper aqueous phase containing the RNA was transferred to a new *eppendorf* RNAse free and 500 μL of isopropanol were added. The *eppendorf* was shaken vigorously and incubated for 10 minutes at room temperature for RNA precipitation. Another centrifugation for 10 minutes at 10600 rpm and 4°C was performed followed by supernatant discharge. The pellet was then washed twice with 1 mL of 75% ethanol. The washed pellet was then air dried and eluted in 30 μL of sterile distilled water (B. Braun, Melsungen, Germany). RNA purity ratios and concentrations were ascertained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA samples were stored at -80°C.

RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue samples

RNA was extracted from 12 μm sections of FFPE primary tumors and the paired metastasis tissues, using a commercially available extraction kit (FFPE RNA/DNA Purification Plus Kit, Norgen Biotek, Thorold, Canada) in accordance with the manufacturer's instructions. Briefly, the process starts with the deparaffinization of the FFPE samples, followed by the digestion with proteinase K [20mg/mL (NZYTECH, Portugal)] and the provided digestion buffer. Following an incubation of 15 minutes at

55°C, the samples are centrifuged and the RNA-containing supernatant is separated from the DNA-containing pellet. Provided buffers and ethanol at 100% are then added to the RNA-containing solution that is then loaded into an RNA Purification Micro Column. The nucleic acids bind to the column and are then washed with provided wash solutions and, finally, eluted in 30 µL of elution solution. RNA purity ratios and concentrations were ascertained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA samples were stored at -80°C.

MicroRNAs cDNA synthesis

cDNA synthesis was performed using miRCURY LNA™ Universal RT miRNA PCR (Exiqon, Vedbaek, Denmark) following manufacturer's instructions. Briefly, all RNA samples previously extracted were first adjusted to a 5ng/µL concentration, using sterile distilled water (B.Braun, Melsungen, Germany). On ice, per each RNase-free PCR tube, it was added: 2 µL of 5x Reaction Buffer, 5 µL of nuclease-free water (Exiqon), 1 µL of enzyme mix and 2 µL of previously concentration-adjusted RNA. Tubes were then vortexed gently and reverse transcription was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) according to the following conditions: 60 minutes at 42°C, followed by 5 minutes at 95°C. Finally, samples were stored at -20ºC.

This kit provides template for all miRNAs in only one reaction and has an amplification step to generate higher quantity of miRNAs (**Figure 5**). The use of Locked Nucleic Acid (LNA)-enhanced primers allows a much higher sensitivity and specificity to the assay.

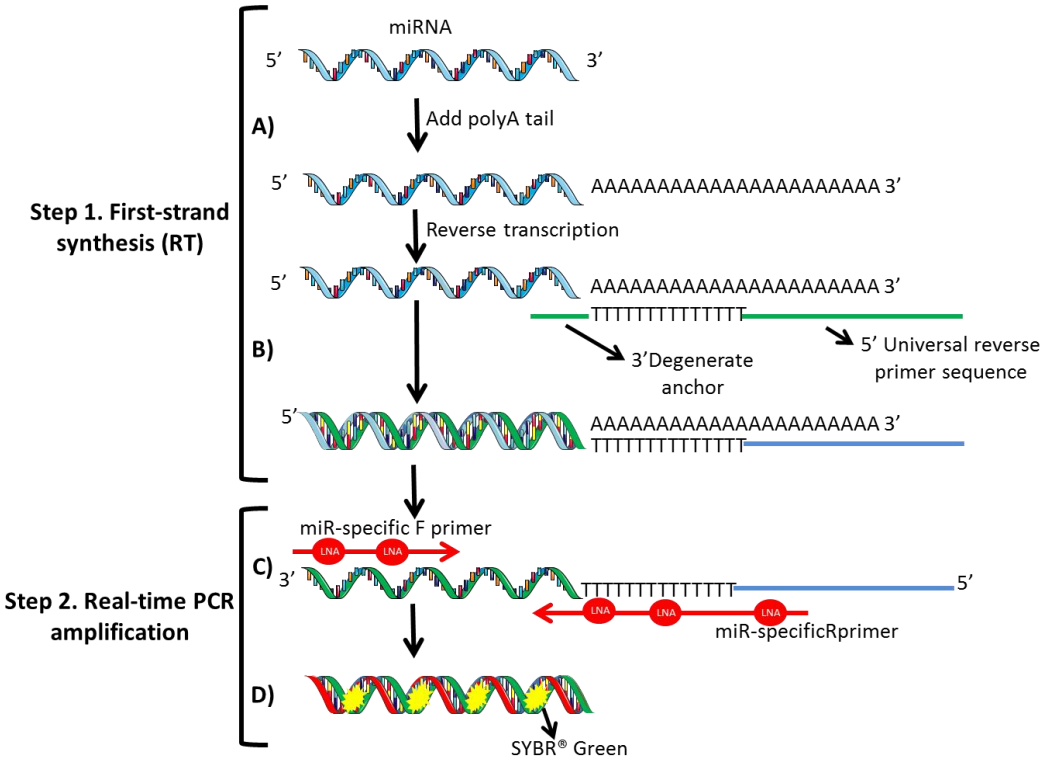

Figure 5. A. A poly-A tail is added to the mature microRNA template. **B.** cDNA is synthetized using a Poly T primer with a 3' degenerate anchor and a 5' universal reverse primer sequence. **C.** The cDNA template is then amplified using microRNA-specific and LNA^{TM}-enhanced forward and reverse primers. **D.** SYBR® Green is used for detection. **Abbreviations:** miRNA – microRNA; miR – microRNA; LNA - Locked Nucleic Acid. Amorim, Maria unpublished.

Global expression assay

Global miRNAs' expression was evaluated using a Cancer Focus miRNA PCR Panel, 384 well (V4.R) (Exiqon). Each plate, besides containing 80 lyophilized LNA™ miRNA primer sets focusing on cancer relevant human miRNAs, also contained interplate calibrators, candidate reference genes (miRNAs and snoRNAs) and one water blank. In each well, it was added 0.05 µL of cDNA previously synthesized, 5 µL of SYBR® Green master mix (Exiqon) and 4.95 µL of nuclease-free water (Exiqon). Quantitative reverse transcription polymerase chain reactions (RT-qPCR) were performed in the LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany). Protocol consisted in incubation for 10 minutes at 95ºC, 45 cycles at 95°C for 10 seconds and 60°C for 1 minute.

Before the analysis, inter-plate calibration (IPC) was performed. First, for each plate, the IPC replicates with standard deviation values higher than 0.5 were eliminated, and the average of the replicates for each plate was calculated, as well as the overall average

(average of IPC values from all plates). The calibration factor was calculated as the difference between plate average and overall average for each plate using the formula:

$$\text{Calibration factor} = IPC_{plate} - IPC_{overall}.$$

Finally, each plate was calibrated by subtracting the calibration factor from all Ct values.

The median values of miR-103a-3p, miR-207, miR-191-5p and SNORD38B were used for normalization, as these genes were the most stably expressed candidate reference genes (data not shown). Differences in expression values for target miRNAs were calculated using the $2^{-\Delta CT}$ method. The selection of deregulated miRNAs for further validation was made considering prominent fold change, good sensitivity for qRT-PCR detection (Ct values, in general, below 30), and novelty.

Individual assays

Initially, cDNA samples from fresh frozen tissues and from FFPE tissues were diluted 80x and 20x, respectively, in sterile distilled water (B. Braun, Melsungen, Germany). Then, on ice, per each well of a 384-well plate it was added: 5 µL of NZYSpeedy qPCR Green Master Mix (2x) (NZYTECH, Portugal), 1 µL of miRNA specific primer mix (miRNA LNA™ PCR primer set, Exiqon), and 4 µL of previously diluted cDNA. Each amplification reaction was performed in triplicate on a LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany). Each plate also contained 2 negative template controls. RT-qPCR protocol consisted in a denaturation step at 95°C for 2 minutes, followed by 40 amplification cycles at 95°C for 5 seconds and 60°C for 20 seconds. Melting curve analysis was performed according to instrument's manufacturer recommendations. In **Table 3** are present the target sequences of miRNAs analyzed in this study.

SNORD38B was used as a reference gene for data normalization, as this gene was the most stably expressed over the whole range of the samples used for the global expression assay. Notwithstanding, the stability SNORD38B expression was empirically validated in more samples before the start of the individual assays. Relative miRNAs expression in each sample was calculated by the $2^{-\Delta CT}$ method, using the formula:

$$\text{Relative expression} = 2^{-\Delta Ct}, \text{ in which } \Delta Ct = Ct_{\text{target miRNA}} - Ct_{\text{reference miRNA}}$$

Table 3. Specific target sequence of miRNAs tested.

Gene	Target sequence
SNORD38B (has)	UCUCAGUGAUGAAAACUUUGUCCAGUUCUGCUACUGACAGUAAGUGAAGAUAAAGU GUGUCUGAGGAGA
hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU
hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-182-5p	UUUGGCAAUGGUAGAACUCACACU
hsa-miR-196a-5p	UAGGUAGUUUCAUGUUGUUGGG
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA
hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG

Methylation Analysis

DNA extraction from fresh frozen tissues

DNA extraction from fresh frozen tissues was performed using the phenol-chloroform method. Twelve-µm frozen sections were cut and placed in 15mL tubes. Then, 2.700 mL of SE buffer (75mM NaCl and 25 mM EDTA), 300 µL of 10% SDS and 25 µL of proteinase K [20mg/mL(NZYTECH, Portugal)] were added to the tissue-containing tubes. Samples were incubated at 55^{O}C and additional proteinase K was added until complete digestion was achieved. Then, all samples were transferred to Phase Lock Light 15mL tubes (5 Prime, Germany) and mixed with 3mL of phenol-chloroform with pH=8 (Sigma-Aldrich, USA). After a centrifugation at 4000 rpm for 20 minutes, the aqueous phases were transferred to new 15mL tubes. For DNA precipitation, 6mL of cold absolute ethanol (Merkmilipore, Germany) and 1mL of ammonia acetate at 7.5M (Sigma-Aldrich, USA) were added to the samples following an overnight incubation at -20ºC. Samples were then centrifuged at 4000 rpm for 20 min and washed twice in 6mL ethanol 70%. The pellets were air dried and eluted in sterile distilled water (B.Braun, Melsungen, Germany). DNA concentration and purity were assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at -20ºC until further use.

Sodium Bisulfite Modification

Sodium bisulfite modification consists on the sulphonation, hydrolic deamination and alkali desulphonation of the unmethylated cytosines that are converted to uracil, while methylated cytosines are refractory to bisulphite-mediated deamination and remain unaltered (175). This protocol allows the differentiation of methylated from unmethylated cytosines upon PCR amplification, where uracil is amplified as thymine while methylated cytosines remain as cytosines. Genomic DNA from all samples and CpGenome™

Universal Methylated DNA (Millipore, USA) were modified using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacturer's instructions. Briefly, the procedure comprised: bisulfite-mediated conversion of 1µg of DNA, using temperature denaturation in the Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) to complement chemical denaturation; binding of the converted single-stranded DNA to the membrane of a Zymo-Spin™ IC Column; washing of the membrane-bound DNA; desulfonation of the membrane-bound DNA; washing to remove the desulfonation agent; elution of the pure converted DNA from the spin column in 60µL or 20µL of sterile distilled water, depending on if it was genomic DNA or CpGenome™ Universal Methylated DNA, respectively. Finally, the modified DNA was stored at -80ºC until further use.

Quantitative Methylation-Specific PCR

Following sodium bisulfite modification, quantitative real-time methylation specific PCR (qMSP) was performed in order to analyze the methylation levels of our samples. qMSP is a quantitative method that combines methylation specific PCR (MSP) and real-time PCR principles, allowing a specific amplification of methylated target DNA and a reference sequence in separate reactions with higher sensitivity. In our study, Actin β (*ACTβ*) was used as a reference gene, allowing to normalize samples for DNA input and also to determine the quality of bisulfite conversion. Besides, the modified CpGenome™ Universal Methylated DNA was used as positive control and it was diluted in five serial dilutions by a 5x dilution factor. These serial dilutions were run in each plate and used to generate a standard curve in order to allow the absolute quantification as well as ascertain PCR efficiency.

Regions enriched for CpG dinucleotides (CpG islands) were predicted using the Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA, USA). The CpG island more distant from the *MIR200B* hairpin coding region was denominated promoter 1 (P1), and the CpG island more proximal to the *MIR200B* hairpin was denominated promoter 2 (P2).

Reactions were performed in 384-well plates using a LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany). Each plate contained all samples in triplicate, the five serial dilutions of the modified positive control in duplicated, and two negative controls. Briefly, per each well were added 1 µL of modified DNA and 5 µL of Xpert Fast SYBR 2X

Master Mix (GRiSP, Porto, Portugal). The primer volume used from a 10mM solution of forward and reverse primers was 0.4 µL for *ACTβ* and miR-200b P1, and 0.2 µL for miR-200b P2. Sterile distilled water was added until 10 µL of reaction volume were achieved. Primer sequences are listed in **Table 4**. PCR program consisted of a period of 3 minutes at 95°C for enzyme activation followed by 40 cycles with 3 seconds at 95°C and 30 seconds at 60/64°C, depending on the primer pair.

For each gene, relative methylation levels were calculated using the formula:

Relative Methylation levels = (Mean quantity of target gene/Mean quantity of *ACTβ*) x 1000

Table 4. Primer sequences and qMSP conditions for each gene studied.

Gene	Sequence	Amplicon	Annealing Temperature (°C)
MIR200B (P1)	F: 5'-GAG CGG AGA TTG GTT AGC-3'	141	60
	R: 5'- TGC AAA ACG ACG AAA CAA TAA -3'		
MIR200B (P2)	F: 5'-TGG ACG TGG TTC GGA TAT AC-3'	121	64
	R: 5'- CGT AGT TTC GGC GAC GTA G -3'		

Statistical Analysis

Comparisons for continuous variables were performed using non-parametric Mann-Whitney U tests. Differences between paired samples were analyzed using a Wilcoxon paired sample test. Fold changes for single miRNAs were calculated using the $2^{-\Delta\Delta CT}$ method (176). Spearman nonparametric correlation test was performed to assess the association between continuous variables. Chi-square test or Fisher's exact test were used as appropriate to compare proportions between two groups.

Receiver Operating Characteristic (ROC) curves were constructed to evaluate miRNAs diagnostic performance and the formula provided in **Table 5** allowed the calculation of specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. The cut-off value established to categorize samples as positive or negative for each miRNA was the highest value obtained by the ROC curve analysis [sensitivity + (1-specificity)]. Logistic regression models were also performed to evaluate the potential of using two miRNAs as a panel to increase their diagnostic performance. Cases positive for both miRNAs were considered positive in the panel.

Table 5. Formulas used for the calculation of the biomarkers performance parameters.

	Tumor	**Normal**
Total	A	B
> cut-off	C	D
< cut-off	E	F
Sensitivity (%)	(C/A) x100	
Specificity (%)	(F/B) x 100	
PPV (%)	(C/(C+D)) x 100	
NPV (%)	(F/(E+F)) x 100	
Accuracy (%)	[(C+F)/(A+B)] x 100	

Abbreviations: PPV - positive predictive value; NPV - negative predictive value.

Some clinicopathological features were grouped, including pT stage (T1&T2 and T3&T4), pN stage (N0&N1 and N2&N3) and grade [grade (G)1&G2 and G3] (34). Age was categorized into four groups (≤44; 45-64; 65-74; ≥75), and miRNA expression levels were categorized according to 25^{th} or 75^{th} percentile. For the survival analysis, Cox-regression univariable and multivariable models were computed to assess standard clinicopathological variables and miRNAs prognostic value. Hazard Ratios (HR) along with respective 95% Confidence Interval (95%CI) were reported. Multivariable Cox models only included the statistically significant variables. Kaplan-Meier with log rank test was used to construct and compare survival curves according to categorized miRNAs expression levels. Endocrine resistance-free survival (ERFS) was defined as the time between surgery and the recurrence dates. Recurrences occurring after 12 months of completing ET were not considered events for this analysis. Disease-free survival (DFS) was defined as the time between surgery date and recurrence date. Distant metastasis-free survival (DMFS) was defined as the time between surgery and the development of distant metastases.

Statistical analysis was performed using SPSS software (SPSS Version 20.0, Chicago, IL) and two-tailed *p*-values were considered statistically significant when $p<0.05$. Graphs were built using GraphPad 6 Prism (GraphPad Software, USA).

RESULTS

Characteristics of study populations

The **discovery cohort**, used for global expression assay analysis, consisted of four luminal A and four luminal B tumors from patients who relapsed, and the same number of patients that did not relapse after adjuvant ET. Patients that relapsed during adjuvant ET or within the first 12 months of completing adjuvant ET were considered endocrine-resistant (**Table 6**).

Table 6. Clinical and pathological data of luminal tumors included in the discovery cohort.

	Molecular Subtype	Age at diagnosis	Grade	Stage	ChT	RT	Recidive Site	Endocrine-resistant
Patients who relapsed	**Luminal A**	82	G2	IIIA	NO	NO	Liver	YES
		41	G3	IIA	YES	YES	Bone	YES
		60	UNKN	IA	NO	YES	Contralateral breast	NO
		43	G2	IIB	YES	YES	Lymph nodes	NO
	Luminal B	65	G3	IIIC	YES	YES	Lung	YES
		63	G2	IIIA	NO	YES	Bone	YES
		67	G2	IIB	NO	NO	Bone	NO
		66	G3	IIIA	NO	NO	Locoregional	NO
Patients who did not relapse	**Luminal A**	70	G3	IIB	NO	YES	n.a.	n.a.
		68	G2	IIB	NO	YES		
		69	G2	IIIA	NO	NO		
		69	G2	IA	NO	YES		
	Luminal B	65	G3	IIIC	YES	YES		
		72	G3	IIIC	NO	YES		
		70	G1	IIB	NO	YES		
		73	G1	IIIC	NO	YES		

Abbreviations: ChT – chemotherapy; RT – radiotherapy; G – grade; UNKN – unknown; n.a. – not applicable.

Overall, 176 fresh frozen tumors and 26 normal breast tissues were included in this study (**Table 7**). Age distribution significantly differed between patients and controls (p=0.003).

Table 7. Clinical and pathological data of tumors and normal breast samples used in this study.

Clinipathological features	Tumor tissues	Normal breast tissues
Patients (n)	176	26
Age median (range)	61 (41-75)	54 (40-70)
Molecular Subtype		
Luminal A	56 (31.8)	n.a.
Luminal B	80 (45.5)	
HER2-enriched	12 (6.8)	
Basal (TNBC)	28 (15.9)	

Abbreviations: HER2 - human epidermal growth factor receptor 2; TNBC – triple-negative breast cancer; n.a. not applicable.

The **validation cohort** was composed of 136 fresh frozen luminal BrCa tissues and 26 normal breast tissues. From the 136 luminal BrCa, 40 derived from patients which

recurred and 96 from patients that did not. Among 40 patients with BrCa recurrence, 22 were considered endocrine-resistant. Clinical and pathological characteristics of patients and controls included in this study are shown in **Table 8**. Endocrine-sensitive and endocrine-resistant groups did not differ significantly concerning age distribution (p=0.127). As expected, among endocrine-resistant BrCa cases, luminal B tumors were more common (p=0.004), and consequently, the same trend was depicted for HER2-positivity (p=0.024) and high Ki-67 index (p<0.001). Moreover, this group also showed more moderate- and high-grade (G2 and G3) BrCa cases (p<0.001). For the remaining clinicopathological features or treatment modalities no significant differences were depicted.

Table 8. Clinical and pathological data of luminal tumors and normal breast samples included in the validation cohort.

Clinipathological features	**Endocrine-sensitive**	**Endocrine-resistant**	**NBr**
Patients (n)	114	22	26
Age median (range)	61.5 (43-73)	60 (41-75)	54 (40-70)
	61.0 (41-75)		
Molecular subtype (%)			
Luminal A	53 (46.5)	3 (13.6)	n.a.
Luminal B	61 (53.5)	19 (86.4)	
Histological type (%)			
Invasive carcinoma of NST (IDC)	99 (86.8)	19 (86.4)	
Invasive lobular carcinoma	6 (5.3)	2 (9.1)	n.a.
Other special subtype carcinoma	1 (0.9)	1 (4.5)	
Mixed type carcinoma	8 (7.0)	0 (0.0)	
Grade (%)			
G1	19 (16.7)	0 (0.0)	
G2	57 (50.0)	9 (40.9)	n.a.
G3	31 (27.2)	11 (50.0)	
Not determined	7 (6.1)	2 (9.1)	
Progesterone receptor *status* (%)			
Positive	96 (84.2)	15 (68.2)	n.a.
Negative	18 (15.8)	7 (31.8)	
HER2 receptor *status* (%)			
Positive	10 (8.8)	6 (27.3)	n.a.
Negative	104 (91.2)	16 (27.3)	
Ki-67 index (%)			
<15%	89 (78.1)	7 (31.8)	n.a.
>15%	20 (17.5)	11 (50.0)	
UNKN	5 (4.4)	4 (18.2)	
Pathological T Stage (%)			
pT1	34 (29.8)	5 (22.7)	
pT2	56 (49.1)	14 (63.6)	n.a.
pT3	3 (2.6)	0 (0.0)	
pT4	5 (4.4)	1 (4.5)	
Not determined	16 (14.0)	2 (9.1)	
Pathological N Stage (%)			
pN0	42 (36.8)	8 (36.4)	
p N1	43 (37.7)	8 (36.4)	n.a.
p N2	9 (7.9)	3 (13.6)	
p N3	5 (4.4)	1 (4.5)	
Not determined	15 (13.2)	2 (9.1)	

Stage (%) I II III Not determined	 17 (14.9) 63 (55.3) 18 (15.8) 16 (14.0)	 3 (13.6) 12 (54.5) 5 (22.7) 2 (9.1)	n.a.
Adjuvant RT Yes No Not determined	 85 (74.6) 19 (16.7) 10 (8.8)	 19 (86.4) 3 (13.6) 0 (0.0)	n.a.
Adjuvant ChT Yes No Not determined	 39 (34.2) 59 (51.8) 16 (14.0)	 12 (54.5) 8 (36.4) 2 (9.1)	n.a.

Abbreviations: NBr – normal breast tissues; HER2 - human epidermal growth factor receptor 2; RT – radiotherapy; ChT – chemotherapy; G – grade; n.a.- not applicable.

Considering paired primary tumors and available metastasis, miRNA expression analysis was performed in 38 tumor samples (16 primary breast tumors and 22 paired metastasis) from 16 luminal patients with paired samples from primary breast tumors and corresponding metastasis (distant, nodal and/or local). The time between diagnosis of primary tumor and metastasis varied from 1.51 to 20.43 years (median 7.27 years). Clinical and pathological characteristics of patients included are shown in **Table 9**.

Table 9. Clinical and pathological data of primary tumor tissues and paired metastasis tissues used in this study.

Patient number	Age at diagnosis	Molecular subtype of primary tumor	Localization of metastasis	Time interval after primary tumor (years)
1	39	Luminal B	Lung	20.43
2	60	Luminal A	Axillary lymph node	16.07
3	36	Luminal B	Bone marrow	3.45
4	35	Luminal B	Liver	11.05
5	74	Luminal B	Pleural	11.75
6	64	Luminal B	Liver	3.54
7	78	Luminal B	Breast Skin	2.73
8	61	Luminal B	Bone	2.76
9	43	Luminal A	Axillary lymph node	11.68
10	55	Luminal B	Breast Skin	6.55
11	51	Luminal A	Lung	6.43
12	63	Luminal B	Pleural	2.90
13	56	Luminal B	Breast skin	3.48
			Axillary lymph node	4.59
14	66	Luminal A	Mediastinum	8.53
			Esophagus	8.93
15	51	Luminal B	Contralateral breast	6.44
			Axillary lymph node	6.52
			Pleural	11.02
			Contralateral breast skin	11.39
16	60	Luminal B	Bone marrow	1.51
			Skin	3.38

Global expression assay analysis

In the global expression assay, one luminal A case with recurrence was excluded from the analysis, due to low RT-qPCR success rate (25% of the miRNAs did not amplify, and the remaining had Ct values higher than 30). Likewise, three (miR-202-3p, -206 and -20b-5p) out of the 80 miRNAs were excluded due to low real-time PCR success rates.

The result of this analysis revealed that, in general, miRNAs had average expression levels lower in the recurrence group compared to the recurrence-free group (data not shown). MiRNAs with fold variation values higher than 1 were selected, resulting in a panel comprising 56 miRNAs (**Appendix VI**).

Gene-specific assays

Assessment of miRNA expression in luminal tumor tissues and normal breast tissues

From the global expression assay analysis, miR-30b-5p, miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p and miR-205-5p were selected for further validation. All these miRNAs disclosed prominent fold change and good sensitivity for qRT-PCR detection. MiR-30b-5p was chosen because most studies focused on other members of the miR-30 family (miR-30f) and, to the best of our knowledge, the predictive potential of miR-30b-5p had not been assessed previously (177-180). Correspondingly, miR-181a-5p and miR-196a-5p were also chosen because their predictive potential in BrCa had not been assessed previously. MiR-200b was selected to confirm the reported association with endocrine-resistance in *in vitro* studies (92, 93). Finally, miR-182-5p was also selected to better ascertain its role in endocrine resistance due to controversial results in global expression assay, since it was overexpressed in luminal B tumors from recurrent patients and downregulated in luminal A tumors from recurrent patients. Furthermore, miR-30c-5p was chosen as a positive control since higher expression levels of this miRNA had been formerly positively associated with benefit of ET, in multivariable analysis, in advanced ER-positive BrCa (181).

To determine “baseline” miRNA expression, 26 normal breast tissues were also analyzed, and we found that miR-181a-5p ($p=0.0007$), miR-182-5p ($p<0.0001$), miR-

196-5p (p<0.0001) and miR-200b-3p (p<0.0001) expression levels were significantly higher whereas miR-205-5p expression levels were significantly lower (p=0.00056) in luminal BrCa tissues. No differences were depicted for the remainder miRNAs (Table 10).

Table 10. MicroRNAs and the respective fold variation values between luminal tumors and normal breast tissues.

miRNA	*p*-value	Fold change ($2^{-\Delta\Delta C_T}$)
miR-30b-5p	0.275	-
miR-30c-5p	0.880	-
miR-181a-5p	0.0007	1.96
miR-182-5p	< 0.0001	5.43
miR-196-5p	< 0.0001	4.73
miR-200b-3p	< 0.0001	7.77
miR-205-5p	0.00056	0.42

Assessment of miRNA expression in non-luminal tumor tissues and evaluation of miRNAs diagnostic performance

To further evaluate the potential of miR-181a-5p, miR-182-5p, miR-196a-5p, miR-200b-3p and miR-205-5p as diagnostic biomarkers, non-luminal tumor tissues (HER2-enriched and basal-like subtypes) were also analyzed. MiR-181a-5p (p=0.0023), miR-182-5p (p<0.0001), miR-196-5p (p=0.0011) and miR-200b-3p (p<0.0001) expression levels were significantly higher in BrCa when compared to normals, whereas miR-205-5p (p<0.001) was significantly lower in tumors comparing with normal breast tissues (**Table 11** and **Figure 6**, left panel).

Table 11. MicroRNAs and the respective fold variation values between luminal tumors and normal breast tissues.

miRNA	*p*-value	Fold change ($2^{-\Delta\Delta C_T}$)
miR-181a-5p	0.0023	1.70
miR-182-5p	< 0.0001	6.45
miR-196-5p	0.0011	3.62
miR-200b-3p	< 0.0001	7.69
miR-205-5p	0.0012	0.36

ROC curves (**Figure 6**, right panel) were constructed and empirical cut-off values were determined for all miRNAs with diagnostic potential.

MiR-182-5p and miR-200b-3p showed the higher Area Under the Curve (AUC) (AUC=0.9696 and AUC=0.9502, respectively). The performance of each miRNA individually was calculated using the empirical cut-off obtained by the ROC curve (**Table 12**).

Table 12. Performance of miRNAs expression as biomarkers for breast cancer detection in tumor tissues.

MiRNA	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-181a-5p	40.909	96.154	98.630	19.380	48.020
miR-182-5p	93.182	92.308	98.795	66.667	93.069
miR-196-5p	53,977	88,462	96,939	22,115	58,416
miR-200b-3p	81.250	96.154	99.306	43.103	83.168
miR-205-5p	76.136	65.385	93.706	28.814	74.752

Abbreviations: PPV - positive predictive value; NPV - negative predictive value.

MiR-182-5p and miR-200b-3p showed the best diagnostic performance. MiR-182-5p showed the highest sensitivity (93.18%), whereas the highest specificity was obtained for miR-200b-3p expression levels (96.15%). However, both had low NPVs. These two miRNAs were combined in panel displaying a diagnostic performance with an AUC of 0.9696 (**Figure 7**), 94.89% sensitivity and 100% specificity. Moreover, a remarkable 100% PPV and 74.29% NPV was also obtained, corresponding to an overall accuracy of 95.55% (**Table 13**).

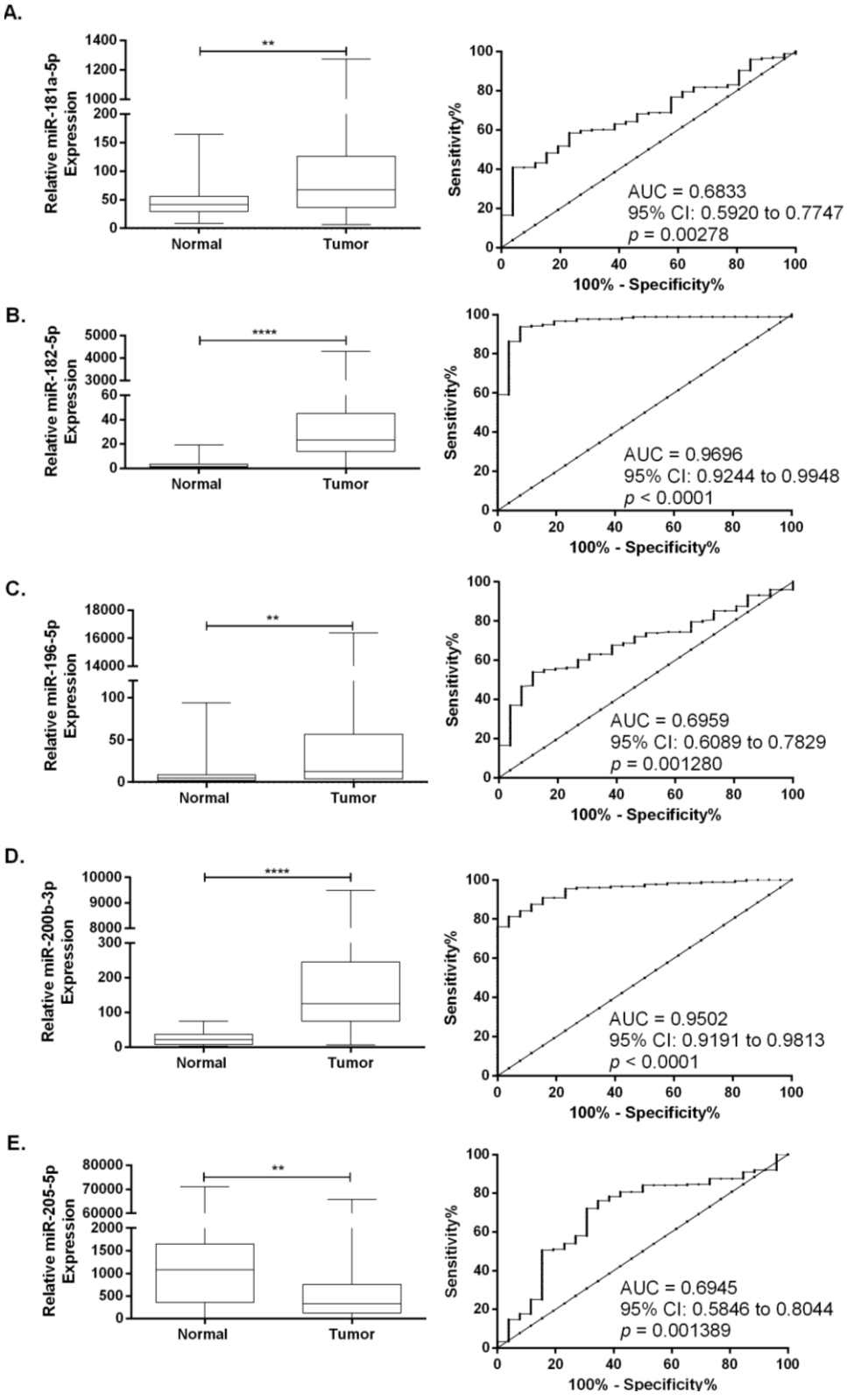

Figure 6. Box-plots (left panel) and the respective Receiver Operating Characteristic (ROC) Curves (right panel) for 182-5p (A), (B), miR-196-5p (C), miR-200b-3p (D) and miR-205-5p (E). A *** denotes p-value <0.001 and a **** denotes p-value < 0.0001 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta CT}$ values multiplied by 1000.

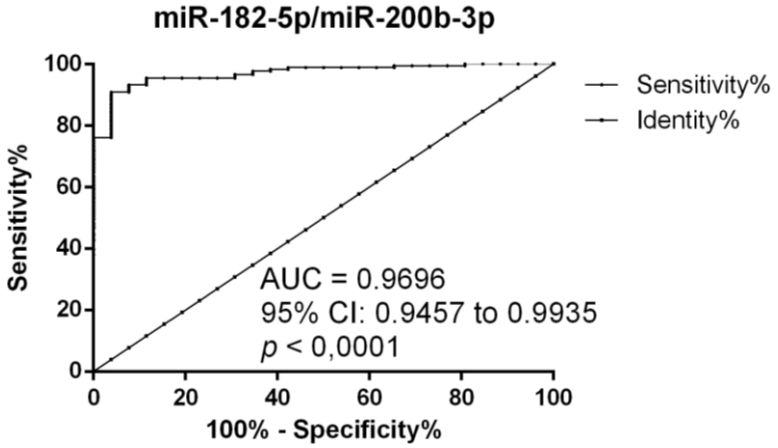

Figure 7. Receiver Operating Characteristic (ROC) Curve for miR-182-5p and miR-200b-3p combined.

Table 13. Performance of miR-182-5p and miR-200b-3p expression levels combined as biomarkers for detection of breast cancer in tumor tissues.

Panel	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-182-5p/miR-200b-3p	94.89	100.00	100.00	74.29	95.55

Abbreviations: PPV - positive predictive value; NPV - negative predictive value.

Validation of selected miRNAs in endocrine-resistant and –sensitive luminal tumor tissues

MiR-30c-5p (p=0.0041), miR-30b-5p (p=0.0396) and miR-200b-3p (p=0.0293) were significantly downregulated in tumor tissues from endocrine-resistant BrCa compared with endocrine-sensitive tumors (**Figure 8**). No differences were depicted for the remainder miRNAs expression levels (**Table 14**).

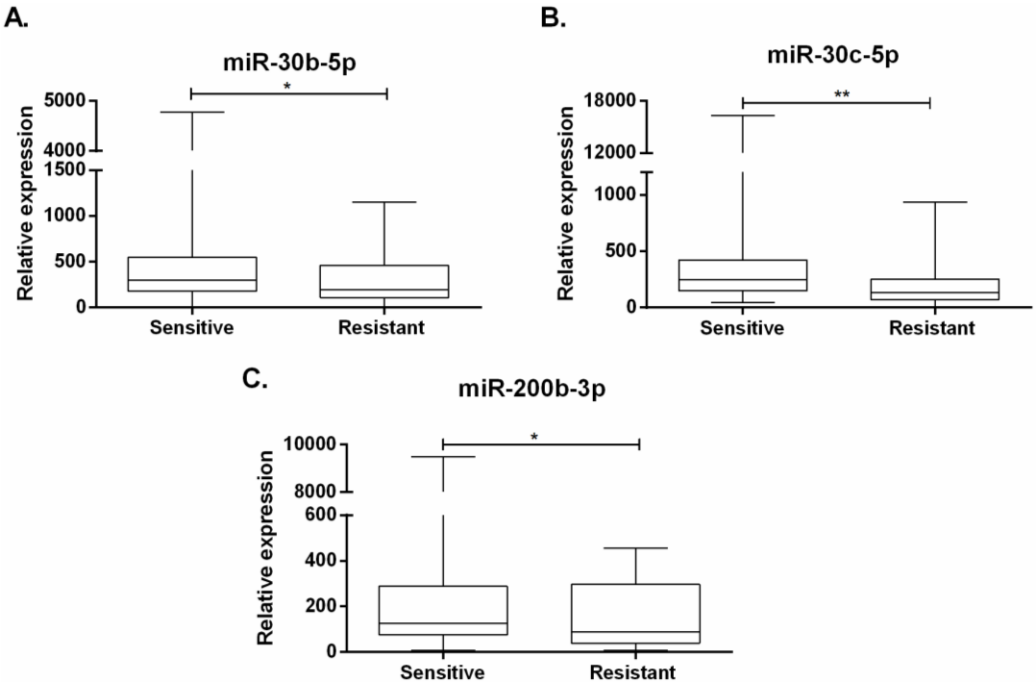

Figure 8. Box-plots of miR-30b-5p (A), miR-30c-5p (B) and miR-200b-3p (C) expression levels in tumor tissues from endocrine-sensitive and –resistant patients. A * denotes *p*-value <0.05 and a ** denotes *p*-value <0.01 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta CT}$ values multiplied by 1000.

Table 14. MicroRNAs and the respective fold variation values between in endocrine-resistant and endocrine-sensitive tumors.

miRNA	*p*-value	Fold change ($2^{-\Delta\Delta CT}$)
miR-30b-5p	0.0396	0.46
miR-30c-5p	0.0041	0.43
miR-181a-5p	0.170	-
miR-182-5p	0.096	-
miR-196-5p	0.995	-
miR-200b-3p	0.0293	0.52
miR-205-5p	0.0565	-

Association between miRNAs expression and clinicopathological features

HER2-negative tumors showed significantly higher miR-30b-5p expression levels (p=0.0447). Additionally, miR-30c-5p expression levels were significantly associated with PR and HER2 *status* (p=0.0314 and p=0.0462, respectively), being higher in PR-positive tumors and in HER2-negative tumors. Moreover, high grade (G3) BrCa displayed significantly higher miR-196a-5p and lower miR-205-5p levels (p=0.0266 and p=0.0268, respectively) compared to G1/G2 BrCa (**Figure 9**).

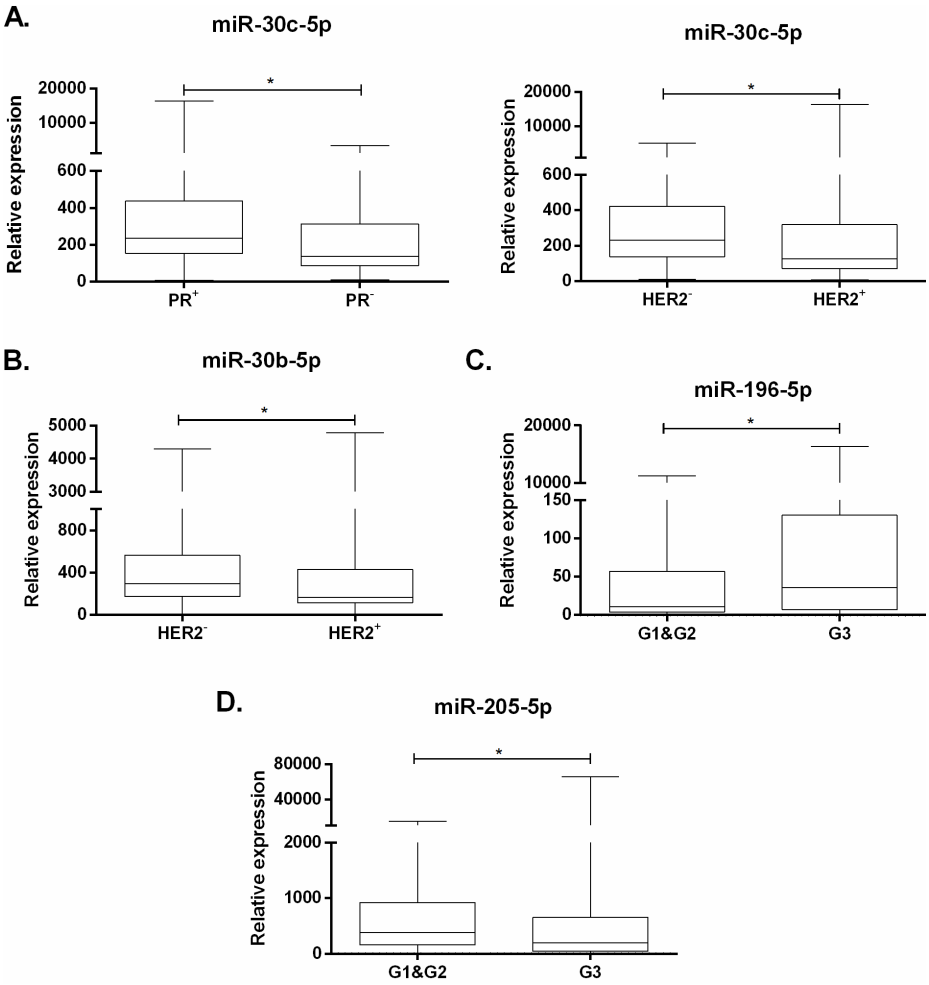

Figure 9. Box-plots of miR-30c-5p (A) expression levels according to PR-status (left) and HER2-status (right), miR-30b-5p (B) expression according to HER2-status, and miR-196a-5p (C) and miR-205-5p (D) expression according to grade. A * denotes p-value <0.05 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta CT}$ values multiplied by 1000.

Survival Analysis

All survival analysis were censured at 15 years of follow-up. The median follow-up time was 121 months (17.6-180 months). At 15 years of follow-up, 79 (58.1%) patients were alive. Of these, 76 patients (55.9%) had no evidence of cancer and 3 patients (2.2%) harboured cancer. Additionally, 57 patients (41.9%) had deceased, 31 of which due to BrCa (22.8%).

In univariable analysis, the majority of the standard clinicopathological parameters significantly associated with ERFS. Specifically, HER2-positivity (HR = 3.46, p=0.010), high Ki-67 index (HR=5.82, p<0.001), high grade (G3) (HR=2.69, p=0.028) and luminal

B subtype (HR=5.11, p=0.009), which disclosed worse ERFS (**Appendix VII**). Furthermore, higher miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p levels predicted better ERFS (**Table 15**, **Figure 10**).

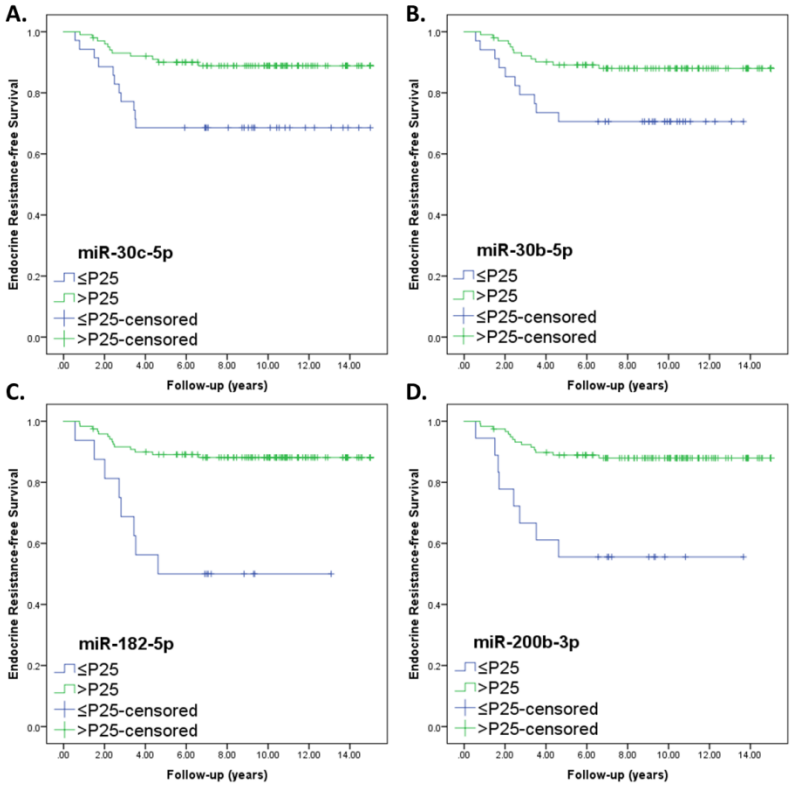

Figure 10. Endocrine Resistance-free survival curves of miR-30b-5p (A), miR-30c-5p (B), miR-182-5p (C) and miR-200b (D). Abbreviations: P25 – percentile 25.

Spearman's correlation was used to assess the correlation between miRNAs expression in tumor tissues in order avoid collinearity/multicollinearity. MiRNAs with the strongest correlation were miR-30b-5p and miR-30c-5p (R=0.842, p<0.001), and miR-200b-3p and miR-182-5p (R=0.837, p<0.001). Besides, miR-30b-5p and miR-30c-5p were also correlated with miR-182-5p (R=0.685, p<0.001 and R=0.638, p<0.001, respectively) and miR-200b-3p (R=0.732, p<0.001 and R=0.730, p<0.001, respectively).

In multivariable analysis, all miRNAs previously identified in the univariable model remained independent predictors of improved ERFS adjusted to molecular subtype. MiRNAs were run in separate analysis to avoid collinearity (**Table 15**).

Table 15. Univariable and multivariable cox regression models assessing the association between microRNAs expression levels and clinical outcome.

Model	Outcome	Variable	HR (95% CI)	*p*-value
Univariable Analysis	**ERFS**	**miR-30b-5p expression categorized** ≤P25 >P25	 1 0.362 (0.156-0.838)	0.018
		miR-30c-5p expression categorized ≤P25 >P25	 1 0.311 (0.135-0.717)	0.006
		miR-182-5p expression categorized ≤P25 >P25	 1 0.194 (0.081-0.464)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.217 (0.091-0.518)	0.001
	DFS	**miR-30b-5p expression categorized** ≤P25 >P25	 1 0.412 (0.208-0.817)	0.011
		miR-30c-5p expression categorized ≤P25 >P25	 1 0.426 (0.223-0.815)	0.010
		miR-182-5p expression categorized ≤P25 >P25	 1 0.213 (0.101-0.452)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.226 (0.110-0.465)	< 0.001
	DMFS	**miR-30b-5p expression categorized** ≤P25 >P25	 1 0.465 (0.224-0.964)	0.040
		miR-30c-5p expression categorized ≤P25 >P25	 1 0.467 (0.234-0.932)	0.031
		miR-182-5p expression categorized ≤P25 >P25	 1 0.284 (0.126-0.644)	0.003
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.287 (0.131-0.628)	0.002
Multivariable Analysis	**ERFS**	**miR-30b-5p expression categorized** [1] ≤P25 >P25	 1 0.367 (1.497-17.112)	0.019
		miR-30c-5p expression categorized [1] ≤P25 >P25	 1 0.353 (0.152-0.818)	0.015
		miR-182-5p expression categorized [1] ≤P25 >P25	 1 0.181 (0.075-0.434)	< 0.001
		miR-200b-3p expression categorized [1] ≤P25 >P25	 1 0.218 (0.091-0.522)	0.001
	DFS	**miR-182-5p expression categorized** [2] ≤P25 >P25	 1 0.194 (0.091-0.415)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	 1 0.246 (0.119-0.511)	< 0.001
	DMFS	**miR-182-5p expression categorized** [3] ≤P25 >P25	 1 0.191 (0.081-0.454)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	 1 0.314 (0.143-0.691)	0.004

[1] Cox regression model adjusted for molecular subtype. [2] Cox regression models adjusted for HER2 *status*.
[3] Cox regression models adjusted for grade and HER2 *status*.

Abbreviations: ERFS – endocrine resistance-free survival; DFS – disease-free survival; DMFS – distant metastasis-free survival; P25 – percentile 25; HR – Hazard ratio; CI – confidence interval.

To disclose the potential of miRNAs expression level as predictors of ERFS for each molecular subtype, a stratified analysis by luminal subtype was performed (**Table 16**). All miRNAs retained statistical significance in luminal B, but not in luminal A lesions.

Table 16. Cox regression models stratified according to the clinicopathological features with statistical significance in the multivariable analysis.

Outcome	Layering Variable	Variable	HR (95% CI)	*p*-value
ERFS	**Luminal A**	**miR-30b-5p expression categorized** ≤P25 >P25	-	0.661
	Luminal B	**miR-30b-5p expression categorized** ≤P25 >P25	1 0.344 (0.140-0.848)	0.020
	Luminal A	**miR-30c-5p expression categorized** ≤P25 >P25	-	0.555
	Luminal B	**miR-30c-5p expression categorized** ≤P25 >P25	1 0.344 (0.140-0.847)	0.020
	Luminal A	**miR-182-5p expression categorized** ≤P25 >P25	-	0.689
	Luminal B	**miR-182-5p expression categorized** ≤P25 >P25	1 0.145 (0.058-0.364)	< 0.001
	Luminal A	**miR-200b-3p expression categorized** ≤P25 >P25	-	0.699
	Luminal B	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.178 (0.071-0.445)	< 0.001
DFS	**HER2-negative**	**miR-182-5p expression categorized** ≤P25 >P25	1 0.179 (0.058-0.364)	0.002
	HER2-positive	**miR-182-5p expression categorized** ≤P25 >P25	1 0.197 (0.058-0.364)	0.004
	HER2-negative	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.235 (0.073-0.750)	0.014
	HER2-positive	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.311 (0.113-0.858)	0.024
DMFS	**Grade 1&2**	**miR-182-5p expression categorized** [1] ≤P25 >P25	1 0.249 (0.076-0.819)	0.022
	Grade 3	**miR-182-5p expression categorized** [1] ≤P25 >P25	1 0.168 (0.044-0.642)	0.009
	HER2-negative	**miR-182-5p expression categorized** [2] ≤P25 >P25	1 0.235 (0.089-0.625)	0.004
	HER2-positive	**miR-182-5p expression categorized**[2] ≤P25 >P25	-	0.053
	HER2-negative	**miR-200b-3p expression categorized** ≤P25 >P25	-	0.066
	HER2-positive	**miR-200b-3p expression categorized** ≤P25	1	0.033

		>P25	0.219 (0.054-0.884)	

[1] Cox regression model adjusted for HER2 *status*. [2] Cox regression models adjusted for grade.
Abbreviations: ERFS - endocrine resistance-free survival; DFS - disease-free survival; DMFS - distant metastasis-free survival; HER2 – human epidermal growth factor 2 receptor; P25 – percentile 25; HR – Hazard ratio; CI – confidence interval.

Regarding DFS, in univariable analysis, HER2-positivity (HR = 3.33, p=0.0002), high Ki-67 index (HR=2.48, p=0.010) and high grade (G3) (HR=2.21, p=0.016) associated with worse DFS (**Appendix VII**). Interestingly, higher miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p expression levels associated with improved DFS (**Table 15**, **Figure 11**).

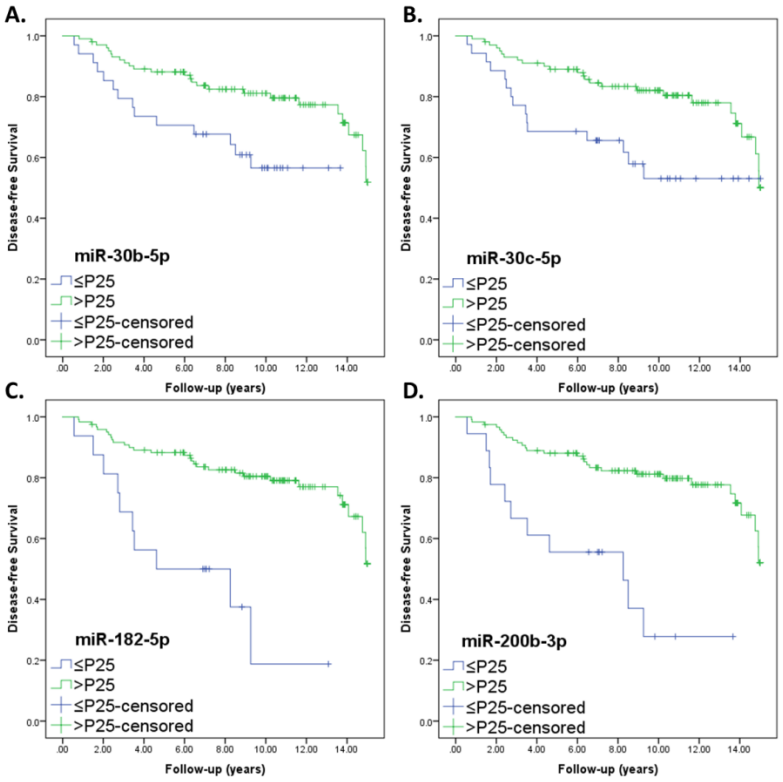

Figure 11. Disease-free survival curves (Kaplan–Meier with log rank test) of miR-30b-5p (A), miR-30c-5p (B), miR-182-5p (C) and miR-200b (D). Abbreviations: P25 – percentile 25.

In the multivariable model, only miR-200b-3p and miR-182-5p were independent prognostic predictors adjusted for HER2 *status* (**Table 15**). After stratifying the analysis according to HER2 *status*, both miRNAs retained statistical significance in both HER2-positive and HER2-negative BrCa (**Table 16**).

Finally, DMFS was also performed, disregarding locoregional recurrences. In line with the results for DFS, in a univariable analysis, HER2-positivity (HR = 3.39, p=0.001),

high Ki-67 index (HR=2.27, p=0.029) and high grade (G3) (HR=2.25, p=0.020) associated with worse DMFS (**Appendix VII**). Besides, higher miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p expression levels also associated with better DMFS (**Table 15**). In multivariable analysis, miR-182-5p retained statistical significance adjusted for HER2 *status* and grade, whereas miR-200b-3p retained statistical significance adjusted for HER2 *status* only (**Table 15**). After stratifying analysis according to HER2 *status* and grade, miR-182-5p retained statistical significance in both low/intermediate and high-grade cancers, as well as in HER2-negative tumors, whereas miR-200b-3p only retained statistical significance in HER2-positive BrCa (**Table 16**).

MicroRNAs' expression analysis in paired metastasis

MiR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p expression levels were further analyzed in primary breast tumors and corresponding metastasis. Only miR-30b-5p and miR-200b-3p expression levels were significantly different between primary tumors and the corresponding metastasis (p=0.008 and p=0.0009, respectively) (**Figure 12**). Namely, miR-30b-5p and miR-200b expression levels were significantly higher in metastatic lesions *versus* primary tumors in 10 of 16 patients and in 11 of 16, respectively, both with a fold variation higher than 1. Conversely, the same was not found for miR-30c-5p and miR-182-5p expression levels (**Figure 13**).

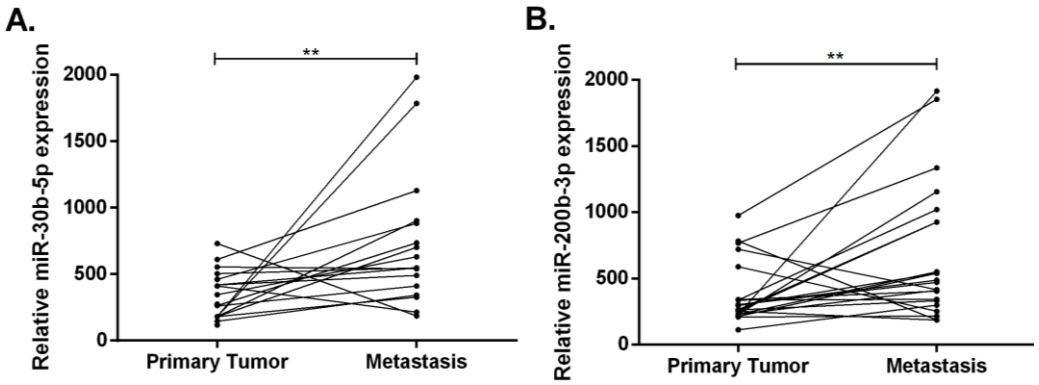

Figure 12. MiR-30b-5p (A) and miR-200b-3p (B) relative expression levels in primary tumors and the corresponding metastasis. A ** denotes *p*-value <0.01 by non-parametric Wilcoxon paired sample test. Y-axis denotes $2^{-\Delta CT}$ values multiplied by 1000.

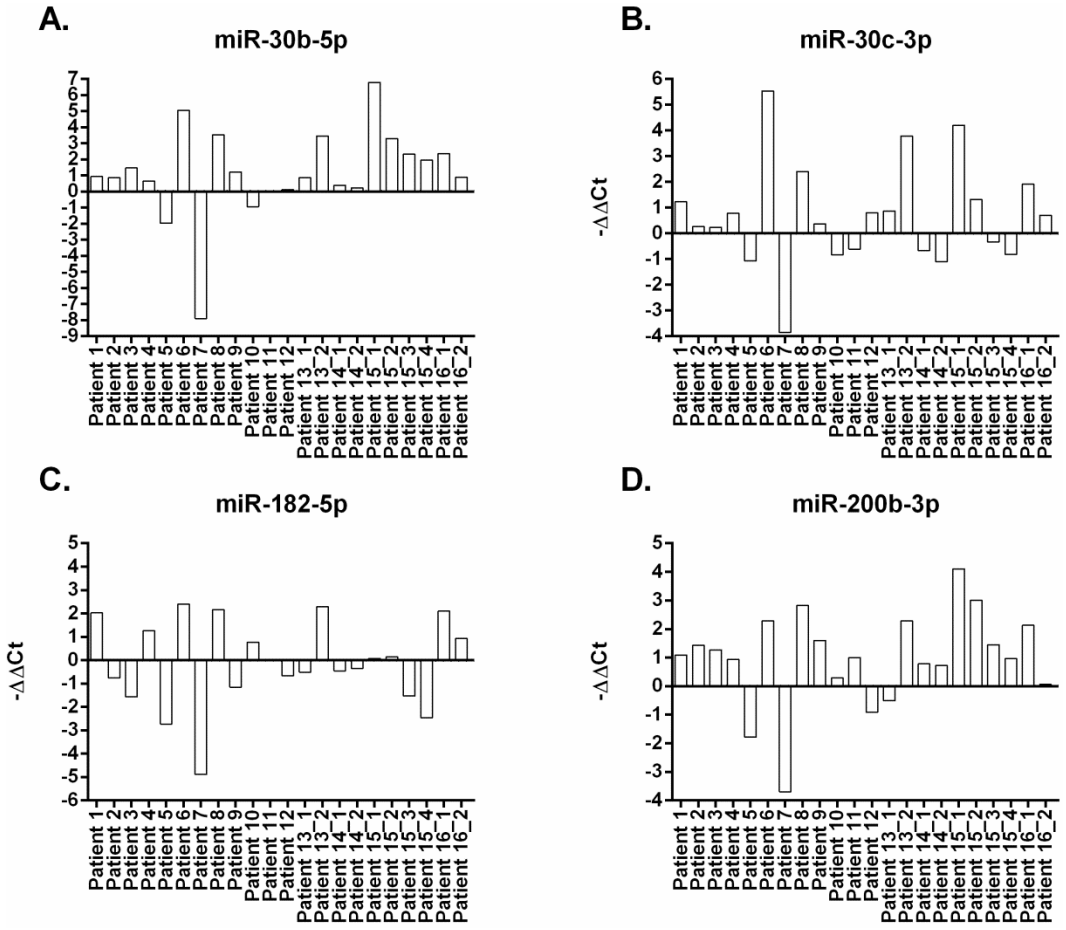

Figure 13. Comparison of miR-30b-5p (A), miR-30c-5p (B), miR-182-5p (C) and miR-200b-3p (D) in primary breast tumors *versus* corresponding metastasis. X-axis represents each patient. Y-axis represents -ΔΔCt values; positive values correspond to higher expression in the distant metastasis *versus* corresponding primary breast tumor.

Methylation Analysis

Since miR-200b-3p downregulation might be due to abnormal DNA methylation, both miR-200b-200a-429 cluster promoter methylation levels were assessed. Thus, methylation analysis was performed in normal breast tissues and in 95 luminal cases [of 136 BrCa luminal cases (18 endocrine-resistant and 77 endocrine-sensitive)] with available DNA.

Globally, P1 methylation levels were significantly higher in luminal BrCa than in normal breast tissues ($p=0.0032$), whereas P2 methylation levels were significantly higher in latter ($p<0.0001$). Moreover, P1 methylation levels only correlated with miR-200b-3p expression in tumor tissues ($R=-0.410$, $p < 0.001$) and P2 methylation levels

significantly correlated with miR-200b-3p expression levels in all breast tissues (R=-0.485, p<0.001) (**Figure 14**).

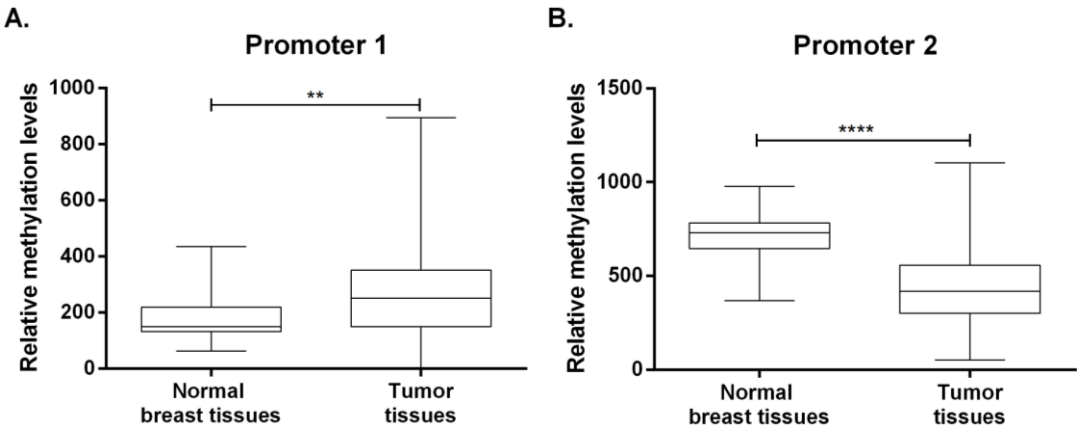

Figure 14. Relative miR-200b-3p promoter 1 (A) and promoter 2 (B) methylation levels in normal breast tissues and tumors. A ** denotes *p*-value <0.01 and a **** denotes a p<0.0001 by non-parametric Mann-Whitney U test. Y-axis denotes relative methylation values multiplied by 1000.

Nonetheless, P1 methylation levels were significantly higher in HER2-negative tumors comparing with HER2-positive tumors (p=0.0358) (**Figure 15**).

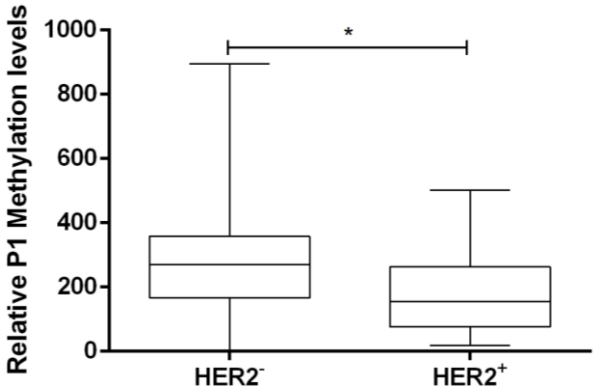

Figure 15. Box-plots of promotor 1 (P1) relative methylation levels in HER2-negative and HER2-positive tumors. A * denotes p-value <0.05. Y-axis denotes relative methylation values multiplied by 1000.

No significant differences were found for methylation levels between endocrine-resistant and –sensitive luminal tumor tissues.

DISCUSSION

BrCa remains the most common malignancy in women and a major cause of morbidity and mortality (1). De-escalation of both systemic and local adjuvant treatment, paralleling trends in surgery, is critical to provide patient-tailored treatment and avoid harmful side effects (32, 182). Indeed, identification of luminal BrCa patients with low-risk for recurrence after or while on ET, for which additional adjuvant systemic treatment can be safely omitted, is very important. Furthermore, the identification of high-risk luminal BrCa patients requiring more aggressive treatment regimens might help avoiding recurrence and subsequent metastatic disease, which currently affects approximately 40% of luminal BrCa patients after adjuvant ET (69, 183). Thus, identification of biomarkers providing predictive and prognostic information in this group of patients is urgently needed. Assessment of specific miRNAs expression deregulation, which has been associated with several mechanisms underlying endocrine resistance or sensitivity (184) might provide such kind of information. Nonetheless, most of those studies have been performed in cancer cell lines and display several limitations, including absence of epithelial-stromal and tumor-host interactions, that could modulate sensitivity *in vivo*. Tissue analysis from patients treated with ET may allow for broader insight into biologically and clinically relevant miRNAs that may serve as markers of response or resistance to ET. Thus, we focused on the identification of aberrantly expressed miRNAs in endocrine-resistant BrCa, exploring its predictive and prognostic value in luminal BrCa patients treated with adjuvant ET.

The first step of this study consisted on the profiling of miRNAs expression patterns, looking for differences between endocrine-sensitive and endocrine-resistant luminal BrCa. Hence, miR-30c-5p, miR-30b-5p, miR-181a-5p, miR-182-5p, miR-196-5p and miR-200b-3p were selected for validation in a larger set of tissue samples.

In addition to luminal tumors, miRNAs expression was also analyzed in normal breast tissues, to determine their “baseline” expression, and in non-luminal tumors, in order to evaluate miRNAs diagnostic potential. Upregulation of miR-182-5p and miR-196-5p and downregulation of miR-205-5p in our cohort of BrCa tissues are consistent with previous publications (185-192), providing indirect validation of our methodological approach. Contrarily, downregulation of miR-200b-3p in tumors compared to normal tissues has been previously reported (193, 194). However, these studies have used as controls normal tissues adjacent to carcinomas, which may not truly represent normal breast tissues. Regarding miR-181a-5p, downregulated levels were reported in BrCa patients’ bodily fluids, however, its overexpression had been also described in other studies for tumorous

breast tissues (195, 196). These inconsistencies in miRNA expression patterns between tumor tissues and bodily fluids might be explained by the currently unknown origin of circulating miRNAs. MiR-182-5p and miR-200b-3p were the miRNAs with the best diagnostic performance. Remarkably, we demonstrated that the combination of these two miRNAs in a panel accurately detected BrCa in tissue samples with 94.89% sensitivity and 100% specificity, corresponding to an overall accuracy of 95.55%. As far as we know, our study is the first reporting the diagnostic performance of these miRNAs in BrCa, although significantly higher miR-182-5p serum levels were already reported in BrCa patients (186).

Considering our main goal, our results have also successfully confirmed the biomarker potential of miR-30c-5p, which was downregulated in endocrine-resistant BrCa patients and independently predicted better ERFS in luminal B BrCa patients. Moreover, miR-30c-5p expression correlated with PR-positivity and HER2-negativity, two of the most important predictive factors for ET sensitivity (42). Interestingly, miR-30b-5p and miR-200b-3p displayed the same trend and together with miR-182-5p, also independently predicted for improved ERFS in luminal B BrCa patients. The lack of significance in luminal A subtype might be due to the small number of cases and events in our series. Importantly, we were able to validate, for the first time in primary BrCa, the association between miR-200b-3p and endocrine-resistance, only previously reported in *in vitro* models (155). Although several members of miR-30f have been reported as markers of favorable prognosis in BrCa (177-180), we revealed that miR-30b-5p might also be predictive of ET response. Finally, concerning miR-182-5p, our results extend previous observations on the correlation with clinical benefit from therapy with tamoxifen in advanced-stage BrCa, but only in univariable analysis (146).

There remains an important question concerning the degree to which the correlation between miRNAs expression is actually a consequence of response to therapies rather than inherently aggressiveness of the tumor. To further evaluate this question, miRNAs prognostic significance was also assessed. Remarkably, we have showed that miR-182-5p and miR-200b-3p are not only predictive, but also independent prognostic markers. Downregulation of these miRNAs associated with decreased DFS in both HER2-positive and HER2–negative BrCa and independently predict DMFS in HER2-negative and HER2-positive cancers, respectively. Because BrCa display higher miR-182-5p and miR-200b-3p levels than normal breast, one may suggest that in early breast carcinogenesis, overexpression of these miRNAs contribute to the emergence of malignant phenotype by

increasing cell proliferation and survival, in line with previous reports from *in vitro* studies (197-199), whereas in a subset of more aggressive BrCa, expression levels decrease, due to yet unknown causes.

The role of miR-200b-3p as a prognostic marker in BrCa is not a novelty (193, 194). Indeed, members of miR-200f are known to act as enforcers of epithelial phenotype through either Zinc finger E-box-binding homeobox (ZEB)-dependent or –independent pathways (200). Intriguingly, most *in vitro* studies consistently attributed an oncogenic role to miR-182-5p (201, 202). Moreover, higher miR-182-5p expression levels were associated with poor clinical outcome in BrCa patients (203), contrarily to our findings. It should be recalled, however, that miR-182 is a member of a miRNA family comprising three homologous, coordinately expressed, miRNAs (miR-183, miR-182 and miR-196) that are clustered in chromosome 7q32.2 and that members of this cluster have been linked to both pro- and anti-metastatic behavior in BrCa, suggesting that miR-183/96/182 cluster members may have divergent functions which are regulated in a context- and tissue-dependent manner (204-206). Furthermore, the 7q32.2 locus has been considered a metastasis suppressor locus, enduring genetic copy number losses in BrCa progression (207). Thus, the association between miR-182-5p downregulation and worse prognosis probably results from a complex molecular scenario and additional studies are required to discriminate which members of the miR-183/96/182 cluster may contribute and to which extent to BrCa prognosis.

MiR-30c-5p, miR-30b-5p, miR-182-5p and miR-200b-3p expression was then evaluated in FFPE metastatic tissues and the paired primary tumors. The stability of miRNAs in FFPE tissues holds enormous potential, especially in BrCa in which late relapses occur frequently. Indeed, it allows miRNA reliable analysis after resistance development and not only just before the therapy, allowing to accurately assess molecular markers across time. MiR-200b-3p and miR-30b-5p expression levels were significantly higher in metastatic tissues when compared to paired primary tumor tissues. As previously mentioned, miR-200b-3p strongly inhibits the early steps of the metastatic process (208, 209). However, metastasis is the result of a multi-step cascade and tumor cells need to undergo widespread modifications to successfully colonize other organs. Indeed, once circulating tumor cells extravasate from the blood vessels, these cells need to recover their epithelial properties by undergoing mesenchymal-to-epithelial transition (MET) (210). Several studies have been shown that miR-200f members are essential in promoting metastatic

colonization, in striking contrast to what happens in the early metastatic steps (211-213). So, the dynamic ability to first undergo EMT and subsequently MET is an important feature of metastatic cells, and miR-200f members modulation might be important in this plastic process. Indeed, it is not the first time that miR-200b expression is reported to be higher in metastatic tissues when compared to primary tumor tissues (211, 214). Regarding miR-30b-5p, its up-regulation in BrCa metastasis has never been reported to date. Nonetheless, other members of the miR-30f have been already linked to EMT in this cancer model (177, 179, 180). Thus, additional studies are needed regarding miR-30b-5p functional role in BrCa cells. The lack of significant differences between primary tumor tissues and the corresponding metastasis for other miRNAs might indicate that the modulation of these miRNAs is not essential for the metastatic process. Nevertheless, it is important to recall that miRNAs expression is highly context- and tissue-dependent, and so, ideally, miRNA expression in normal metastasis-host tissues should also be assessed. We could then be able to ascertain if the differential expression of miRNAs in the primary tumors *versus* metastatic tissues are a consequence of their modulation in the metastasis-host tissue. Indeed, we have observed that some metastasis of the same patient from different locations have different expression patterns.

The exploitation of the mechanisms by which miRNAs might be deregulated in BrCa may deepen our understanding on their potential role and functional implications. An analysis of the literature revealed that the cluster in which miR-200b-3p is included - miR-200b-200a-429 – has two promoter regions with CpG enrichments (215). Furthermore, *in vitro* studies, found that the treatment of an endocrine-resistant BrCa cell line with the demethylation agent 5-aza-2'-deoxycytidine increased miR-200b expression (155). Therefore, we sought to determine whether miR-200b-3p plastic expression in luminal BrCa is regulated by aberrant DNA methylation. Overall, our results suggest that miR-200b P1 promoter methylation might be malignant-specific, while miR-200b P2 might be tissue-specific. Besides, miR-200b overexpression in tumor tissues might be due to the loss of P2 methylation levels in tumor tissues. However, no methylation differences were found for both promoters between endocrine-resistant and -sensitive tumors, suggesting that miR-200b-3p downregulation in endocrine-resistant tumors might be dependent on additional mechanism, such as the recruitment of repressive histone marks. Indeed, recent *in vitro* studies have suggested that in cancer cells, the miR-200b cluster is more prone to be silenced primarily through polycomb group-mediated H3K27me3 gene silencing than by DNA methylation (216, 217). MiR-200b cluster P1 and P2 methylation levels have been

previously analyzed in breast clinical samples in another study, in which higher P2 methylation levels were similarly associated with HER2 positivity (218). Nevertheless, we firstly showed that miR-200b promotors' methylation levels are differentially methylated in normal breast and BrCa tissues.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the recent achievements in the last years concerning the role of miRNAs in endocrine resistance, the implementation of miRNAs for clinical use remains at an early stage. Indeed, we have verified that there are widespread inconsistencies across several studies regarding miRNAs expression and functional role that might be attributable to differences in the type of biological samples under investigation, differences in the methodologies used and differences in the genes used for data normalization. These discrepancies highlight the need to standardize experimental conditions, as well as the need to validate the findings in additional independent cohorts before its clinical utility may be established in daily clinical practice.

In this master dissertation, we have showed that miRNAs might be suitable markers for BrCa management. Indeed, miR-182-5p and miR-200b-3p combined in a panel demonstrated high sensitivity, specificity and accuracy for BrCa diagnosis. Additionally, these miRNAs were also shown to be independent prognostic factors. Moreover, miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p independently predicted longer ERFS in luminal B BrCa patients. Overall, our results suggest that a panel of miRNAs might be tested in primary tumor tissues to assess the likelihood of recurrence and resistance to ET in newly diagnosed luminal BrCa. Nevertheless, these miRNAs need to be carefully validated, ideally in multicenter studies, to generate more conclusive results. Furthermore, *in vitro* studies, including gain and loss of function assays following *in vitro* treatment with ET, are also critical to functionally characterize the role of these miRNAs.

Thus, in near future, we intend to increase the number of luminal A tumors to accurately evaluate the predictive potential of these miRNAs in this molecular subtype.

Besides, we have verified that miR-30b-5p and miR-200b-3p modulation might be important during the metastatic cascade, e.g. for the ability to first undergo EMT in the early metastatic steps and subsequently MET for successful colonization. Consequently, we plan to increase our cohort of primary tumors and paired metastasis.

Moreover, it would be very interesting to assess the expression of these miRNAs in liquid biopsies, evaluating their potential as non-invasive biomarkers. Indeed, miRNAs in circulation would enable the noninvasive monitoring of the disease during the course of the treatment, which might allow the detection of resistance to ET at an early stage, improving the care of luminal BrCa patients.

Finally, we have hypothesized that the dynamic regulation of miR-200b-3p might be dependent on other epigenetic mechanisms rather than DNA methylation, which will be further explored using *in vitro* models.

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SUPPLEMENTARY MATERIAL

Appendix I. Magnitude of risk of BrCa risk and protective factors with different scientific evidence. Adapted from (219).

Characteristic	Menopausal Status *	Comparison Category	Risk Category	Estimate of Effect #
Traditional Risk Factors for Breast Cancer				
Age at menarche	Both	Absence of the Factor	Greatest Risk Category	OR 1.3
Age at first live birth				OR 1.9
Age at menopause				OR 1.5
Family history of breast cancer in first-degree relatives				OR 1.7 (mother) OR 5.0 (two first-degree relatives)
Proliferative benign breast disease				OR 2.0 OR 5.0 (atypical hyperplasia)
Lobular carcinoma *in situ*				OR 10
Birthplace/ethnicity				OR 1.5–2.5
Newer Epidemiologic Risk Factors for Breast Cancer				
Demographic Factors				
Age (yr)	Both	40–44	50–54	IRR 2.09
			75–79	IRR 4.11
Race		African American	White	IRR 1.16
		Asian/Pacific Islander		IRR 1.42
		Hispanic		IRR 1.57
Genetic Factors				
BRCA1 mutation	Both	No mutation	Mutation	Lifetime risk 50–73% by age 50 and 65–87% by age 70
BRCA2 mutation				Lifetime risk 59% by age 50 and 82% by age 70
Hormonal Factors				
Oral contraceptive use	Both	Never users	Current users	RR 1.24 (1.15–1.33)
			≥10 yr since last use	RR 1.01 (0.96–1.05)
Postmenopausal hormone therapy use	Postmenopausal	Nonusers with an intact uterus	Estrogen and progestin users	HR 1.24 (1.01–1.54)
		Nonusers with a hysterectomy	Estrogen users	HR 0.80 (0.62–1.04)
Circulating estradiol	Premenopausal Postmenopausal	Lowest quartile Lowest quintile	Highest quartile Highest quintile	OR 1.00 (0.66–1.52) RR 2.00 (1.47–2.71)
Circulating estrone	Premenopausal Postmenopausal	Lowest quartile Lowest quintile	Highest quartile Highest quintile	RR 2.00 (1.47–2.71) RR 2.19 (1.48–3.22)
Testosterone	Premenopausal Postmenopausal	<1.13 nmol/L Lowest quintile	≥2.04 nmol/L Highest quintile	OR 1.73 (1.16–2.57) RR 2.22 (1.59-3.10)
Other Biologic Factors				
Mammographic breast density	Both	<5% density	≥75% density	RR 4.64 (3.64–5.91)
Bone mineral density	Postmenopausal	Lowest quartile at each of three skeletal sites	Highest quartile at each of three skeletal sites	RR 2.70 (1.4–5.3)
Circulating IGF-1	Premenopausal Postmenopausal	25^{th} percentile	75^{th} percentile	OR 1.93 (1.38–2.69) OR 0.95 (0.62–1.33)

Circulating IGFBP-3	Premenopausal Postmenopausal	25th percentile	75th percentile	OR 1.96 (1.28–2.99) OR 0.97 (0.53–1.77)
Behavioral Factors				
Body mass index	Postmenopausal	<21.0 kg/m^2	≥33.0 kg/m^2	≥33.0 kg/m^2
Height	Premenopausal Postmenopausal	<1.60 cm	≥1.75 cm	RR 1.42 (0.95–2.12) RR 1.28 (0.94–1.76)
Weight	Postmenopausal	<60.0 kg	≥80.0 kg	RR 1.25 (1.02–1.52)
Alcohol use	Both	Never drinkers	>12 g/day	RR 1.10 (1.06–1.14)
Smoking	Postmenopausal	Never smokers	Smoked > 40 yr	RR 1.5 (1.2–1.9)
Night work	Both	No nightshift work	Any nightshift work	OR 1.48 (1.36–1.61)
Dietary Factors				
Total fat intake	Both	Lowest quartile	Highest quartile	OR 1.13 (1.03–1.25)
Saturated fat intake				OR 1.19 (1.06–1.35)
Meat intake				OR 1.17 (1.06–1.29)
Environmental Factors				
Ionizing radiation	Both	0–0.09 Gy exposure to Nagasaki or Hiroshima atomic bomb	≥0.50 Gy exposure to Nagasaki or Hiroshima atomic bomb	RR = 9 at age 0–4 RR = 2 at age 35–39
Possible Protective Factors for Breast Cancer				
Biologic Factors				
Bone fracture	Postmenopausal	No fracture in past 5 yr	History of fracture	OR 0.80 (0.68–0.94)
Behavioral Factors				
Body mass index	Premenopausal	<21.0 kg/m^2	≥33.0 kg/m^2	RR 0.58 (0.34–1.00)
Physical activity		<9.1 hr/wk	≥20.8 hr/wk	OR 0.74 (0.52–1.05)
NSAID use	Both	Nonusers	Current user of any NSAID	OR 0.80 (0.73–0.87)
Dietary Factors				
Calcium (dietary)	Postmenopausal	≤500 mg/day	>1250 mg/day	RR 0.80 (0.67–0.95)
Folate (total)	Both	150–299 µg/day	≥600 µg/day	RR 0.93 (0.83–1.03)
Soy	Premenopausal Postmenopausal	Low intake	High intake	OR 0.70 (0.58–0.85) OR 0.77 (0.60–0.98)
Vitamin D (total)	Postmenopausal	<400 IU	≥800 IU	RR 0.89 (0.77–1.03)

[*]Menopausal status at the time of diagnosis. [#] 95% confidence intervals are given in parentheses. **Abbreviations:** HR - hazard ratio; IGF-1 - insulin-like growth factor-1; IGFBP-3 - insulin-like growth factor-1 receptor binding protein-3; IRR - incidence rate ratio; OR - odds ratio; RR -relative risk; NSAID - nonsteroidal anti-inflammatory drug; yr – years.

Appendix II. Nottingham combined histologic grade. Adapted from (36).

Criteria	Score		
	1	**2**	**3**
Glandular/Tubular differentiation	>75 %	10-75 %	<10 %
	of tumor area forming glandular/ tubular structures		
Nuclear pleomorphism	Nuclei small with little increase in size in comparison with normal breast epithelial cells, regular outlines, uniform nuclear chromatin, little variation in size	Cells larger than normal with open vesicular nuclei, visible nucleoli, and moderate variability in both size and shape	Vesicular nuclei, often with prominent nucleoli, exhibiting marked variation in size and shape, occasionally with very large and bizarre forms
Mitotic Counting	≤7 HPF	8-14 HPF	≥15 HPF
Total Score	3-5 **Grade 1** (Well differentiated)	6 or 7 **Grade 2** (Moderately differentiated	8 or 9 Grade 3 (Poorly differentiated)

Abbreviations: HPF – High-power field

Appendix III. Tumor-node-metastases (TNM) staging system for carcinoma of the breast. Adapted from (220).

TNM staing system		
Primary tumor (T)		
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
Tis	Carcinoma in situ	
	DCIS	Ductal carcinoma in situ
	LCIS	Lobular carcinoma in situ
	Paget's	Paget's disease (Paget disease) of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget's disease are categorised based on the size and characteristics of the parenchymal disease, although the presence of Paget's disease should still be noted.
T1	Tumour ≤20 mm in greatest dimension	
	T1mi	Tumour ≤1 mm in greatest dimension
	T1a	Tumour >1 mm but ≤5 mm in greatest dimension
	T1b	Tumour >5 mm but ≤10 mm in greatest dimension
	T1c	Tumour >10 mm but ≤20 mm in greatest dimension
T2	Tumour >20 mm but ≤50 mm in greatest dimension	
T3	Tumour >50 mm in greatest dimension	
T4	Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)	
	T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
	T4b	Ulceration and/or ipsilateral satellite nodules and/or oedema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
	T4c	Both T4a and T4b

	T4d	Inflammatory carcinoma
Regional lymph nodes (N)		
Clinical (cN)		
cNX	Regional lymph nodes cannot be assessed (e.g. previously removed)	
cN0	No regional lymph node metastases	
cN1	Metastases to movable ipsilateral level I, II axillary lymph node(s)	
cN2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases	
	cN2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
	cN2b	Metastases only in clinically detectedk ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
cN3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detectedk ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement	
	cN3a	Metastases in ipsilateral infraclavicular lymph node(s)
	cN3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
	cN3c	Metastases in ipsilateral supraclavicular lymph node(s)
Pathological (pN)		
pNX	Regional lymph nodes cannot be assessed (e.g. previously removed or not removed for pathological study)	
pN0	No regional lymph node metastasis identified histologically	
	pN0(i−)	No regional lymph node metastases histologically, negative immunohistochemistry (IHC)
	pN0(i+)	Malignant cells in regional lymph node(s) not >0.2 mm [detected by haematoxylin and eosin (H&E) staining or IHC including isolated tumour cell clusters (ITCs)]
	pN0(mol−)	No regional lymph node metastases histologically, negative molecular findings (RT-PCR)
	pN0(mol+)	Positive molecular findings (RT-PCR), but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in one to three axillary lymph nodes; and/or in internal mammary nodes with metastases detected by SLNB but not clinically detected	
	pN1mi	Micrometastases (>0.2 mm and/or >200 cells, but none >2.0 mm)
	pN1a	Metastases in one to three axillary lymph nodes, at least one metastasis >2.0 mm
	pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by SLNB but not clinically detectedl
	pN1c	Metastases in one to three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SLNB but not clinically detectedl
pN2	Metastases in four to nine axillary lymph nodes; or in clinically detectedk internal mammary lymph nodes in the absence of axillary lymph node metastases	
	pN2a	Metastases in four to nine axillary lymph nodes (at least one tumour deposit >2.0 mm)
	pN2b	Metastases in clinically detectedk internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in ≥10 axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected ipsilateral internal mammary lymph nodes in the presence of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SLNB but not clinically detected; or in ipsilateral supraclavicular lymph nodes	

	pN3a	Metastases in ≥10 axillary lymph nodes (at least one tumour deposit >2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
	pN3b	Metastases in clinically detectedk ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SLNB but not clinically detectedl
	pN3c	Metastases in ipsilateral supraclavicular lymph nodes
Distant metastasis (M)		
M0	No clinical or radiographic evidence of distant metastases	
	cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumour cells in circulating blood, bone marrow or other non-regional nodal tissue that are not >0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven >0.2 mm	

Appendix IV. Stage grouping system for carcinoma of the breast. Adapted from (220).

Anatomic stage/prognostic groups	T	N	M
Stage 0	Tis	0	0
Stage IA	1	0	
Stage IB	0	1mi	
	1	1mi	
Stage IIA	0	1	
	1	1	
	2	0	
Stage IIB	2	1	
	3	0	
Stage IIIA	0	2	
	1	2	
	2	2	
	3	1	
	3	2	
Stage IIIB	4	0	
	4	1	
	4	2	
Stage IIIC	Any T	3	
Stage IV	Any T	Any N	M1

Appendix V. Amorim, Maria, et al. "Decoding the usefulness of non-coding RNAs as breast cancer markers." Journal of translational medicine 14.1 (2016): 265.

Decoding the usefulness of non-coding RNAs as Breast Cancer markers

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Decoding the usefulness of non-coding RNAs as Breast Cancer markers

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ABSTRACT

Although important advances in the management of breast cancer (BC) have been recently accomplished, it still constitutes the leading cause of cancer death in women worldwide. BC is a heterogeneous and complex disease, making clinical prediction of outcome a very challenging task. In recent years, gene expression profiling emerged as a tool to assist in clinical decision, enabling the identification of genetic signatures that better predict prognosis and response to therapy. Nevertheless, translation to routine practice has been limited by economical and technical reasons and, thus, novel biomarkers, especially those requiring non-invasive or minimally invasive collection procedures, while retaining high sensitivity and specificity might represent a significant development in this field. An increasing amount of evidence demonstrates that non-coding RNAs (ncRNAs), particularly microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), are aberrantly expressed in several cancers, including BC. miRNAs are of particular interest as new, easily accessible, cost-effective and non-invasive tools for precise management of BC patients because they circulate in bodily fluids (e.g., serum and plasma) in a very stable manner, enabling BC assessment and monitoring through liquid biopsies. This review focus on how ncRNAs have the potential to answer present clinical needs in the personalized management of patients with BC and comprehensively describes the state of the art on the role of ncRNAs in the diagnosis, prognosis and prediction of response to therapy in BC.

Key words: Biomarkers, microRNA, Long nonconding RNA, Diagnostic, Prognostic

BACKGROUND

Breast cancer (BC) is one of the most common cancers with more than 1,300,000 cases diagnosed and 450,000 deaths occurring each year, worldwide [1]. Due to earlier diagnosis and implementation of adjuvant chemo- and hormone-therapies (HT), BC mortality has been declining, although it remains the most common cause of cancer-related death among women [2]. At present, most patients are diagnosed at localized disease stage, but 20-85% of all patients will eventually develop recurrent and/or metastatic disease [3].

BC is intrinsically heterogeneous, representing a spectrum of diseases with distinct morphology, molecular traits, prognosis, and therapeutic options. On the basis of gene expression, BC cases are often classified into one of five intrinsic subtypes [4]. The large majority of estrogen receptor (ER) and/or progesterone receptor (PR)-positive ($^{+}$) tumors are of the luminal subtypes that typically express luminal cytokeratins (CK) 8 and 18 [5]. These tumors are further subdivided into Luminal A and Luminal B, according to the expression levels of Ki67, a nuclear protein that is associated with cellular proliferation. The ER and PR-negative ($^{-}$) tumors are divided into three subtypes: the basal-like subtype, characterized by the expression of CK 5/6 and CK17; the human epidermal growth factor receptor 2 (HER2)-enriched subtype, which are positive for HER2; and the "normal-like" subtype, characterized by a similar gene expression pattern as the normal breast. This last subtype remains enigmatic as to whether it represents a separate subtype or a technical artifact introduced by the contamination of cancerous cells with their surrounding normal tissue [5].

BC clinical decisions are based on routine assays for ER, PR and HER2, as well as Ki67 [6]. The molecular phenotype of the tumor is indicative of the most suitable treatment, i.e., either endocrine therapy (ET) in hormone receptor positive or HER-targeted therapy in $HER2^{+}$ tumors [7]. Globally, ER^{-} tumors have a poorer prognosis in the first few years after diagnosis, but after 5 to 10 years, ER^{+} tumors demonstrate the poorest outcome [8].

However, not all ER^+ BCs behave similarly, and the studies conducted in recent years show that luminal A and B BCs should be perceived as distinct entities [9]. Luminal A subtype has been shown to exhibit good clinical outcomes with ET whereas the pattern of mortality rates associated with the luminal B tumors is similar to those of the non-luminal subtypes [10]. However, Luminal A, the most frequently occurring BC subtype, is also the most heterogeneous subtype, both molecularly and clinically [11]. Indeed, ER expression itself fails to predict which ER^+ tumors will respond or be resistant to different modalities of ET, and resistance has been reported in 30% of ER^+ BCs [12].

Due to molecular heterogeneity, clinical decisions based solely upon histopathologic analysis or one or small numbers of genes or their coding proteins in the tumor tissue are limited. Moreover, the widespread use of gene-expression profiling using commercially available molecular signatures for the examination of multiple expressed genes is also of limited application, primarily due to the cost and to reproducibility issues [13,14].

Recently, several studies have reported on the association between microRNAs (miRNAs) and BC, suggesting its usefulness as disease biomarkers. Interestingly, miRNA detection in bodily fluids appear to have superior accuracy than messenger RNA (mRNA) profiling because of their high tissue-specificity, stability, and aberrant expression in different tumor types [15]. MiRNAs have the additional advantage of being long-lived *in vivo* [16] and very stable *in vitro* [17,18], which might be critical in a clinical setting. Indeed, tumor cells were suggested to release miRNAs stabilized by their association with RNA-binding proteins and by their incorporation into microvesicles [19,20].The emergence of non-coding RNA (ncRNAs) as biomarkers may add robustness to the current molecular classification of human BC, with the potential for improving diagnosis and monitoring of BC. Thus, in this review, we will focus on ncRNAs as potential diagnostic, predictive and prognostic biomarkers for BC management.

Evidence Acquisition

For the selection of bibliography, PubMed publications on BC were searched using the keywords breast cancer, noncoding RNAs and microRNAs. References of all articles were also examined for additional potentially relevant studies. The criteria for article selection were: written in English, central theme based on ncRNAs and BC. Original reports were selected based on the detail of analysis, mechanistic support of data, novelty, and potential clinical usefulness of the findings.

NON-CODING RNAS

It is currently acknowledged that at least 98% of the mammalian genomes and other complex organisms are transcribed into ncRNAs [21]. In fact, ncRNAs that were previously thought to be “transcription noise”, are believed to be a hidden layer of internal signals that control various levels of gene expression, playing a significant role in cell homeostasis and its deregulation is involved in the development of several human diseases. The family of ncRNAs, in addition to the well know transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nucleolar RNAs (snoRNAs), includes the recently discovered long noncoding RNAs (lncRNAs) and miRNAs.

Transfer RNA (tRNA)

tRNAs are small ncRNA transcripts, typically with 76 to 90 nucleotides (nt) in length, that serve as physical link between mRNA and the aminoacid sequence of proteins [22]. In 2009, Pavon-Eternod [23] analyzed genome-wide tRNA expression and found that tRNAs were increased in BC compared to normal breast tissues. Their results also suggested a functional consequence of tRNA over-expression in tumor cells, which seems to be selective and may increase the translational efficiency of genes relevant to cancer development and progression.

Recent studies indicated that precise cleavage of tRNAs generate active products [24]. Indeed, high levels of tRNA-derived miRNas or of tRNA-derived molecules termed 5'tRNA halves are likely to be a manifestation of tRNA over-expression. Park [25] reported that miR-1280 - a tRNA-derived fragment - was significantly up-regulated in blood of BC patients, particularly in metastatic BC patients, compared to healthy subjects and decreased significantly after systemic treatment in patients who responded to treatment, while increasing in the blood of patients with non-responding tumors. Moreover, BC is associated with expression deregulation– either increase or decrease – in the circulating levels of 5'tRNA halves derived from specific tRNA isoacceptors [26], and changes in circulating 5'tRNA halves were associated with specific tumor features, such as ER/PR/HER2-status, raising the possibility of a causal connection with some aspects of breast carcinogenesis.

Long noncoding RNAs (lncRNAs)

LncRNAs are ncRNA molecules usually longer than 200 nts that do not fit into known classes of small or structural RNAs, and that may function as either primary or spliced transcripts [27]. LncRNAs may be transcribed from various genomic locations, as well as in their own stand-alone position in the genome - long intergenic non-coding RNAs (lincRNAs) [28]. LncRNAs have gained widespread attention in recent years as a potentially new and crucial layer of biological regulation, controlling cell cycle, apoptosis and differentiation by acting as protein-DNA or protein-protein scaffolds, miRNA sponges, protein decoys, and regulators of translation [29].

LncRNAs in breast cancer

LncRNAs were already found to be differentially expressed in BC tissues compared to normal breast tissues and recent studies have demonstrated their key regulatory role in gene expression and BC biology through diverse mechanisms [30].

Diagnostic biomarkers

Expression levels of lncRNAs have been investigated in BC tissues compared to normal tissues indicating that some may be potential biomarkers for BC diagnosis. Ding et al found that lincRNA-BC2 and lincRNA-BC5 were consistently up-regulated (more than 2-fold) in BC samples, whereas lincRNA-BC4 and lincRNA-BC8 were down-regulated [31]. Moreover, lincRNA-BC4 expression was significantly lower in grade III BC, and lincRNA-BC5 expression was significantly higher in grade III, whereas lincRNA-BC2' expression significantly associated with lymph node metastasis (LNM). Remarkably, lncRNAs' expression was also found to be highly associated with BC subtype classification [32]. Later studies have also demonstrated that lncRNAs are amenable for detection in bodily fluids. For example, the serum expression levels of circulating lncRNA RP11-445H22.4 were found significantly increased in BC patients, identifying BC cases with 92% sensitivity and 74% specificity [33].

Prognostic biomarkers

In addition to lncRNAs potential use as diagnostic biomarkers, they have been suggested as valuable prognostic biomarkers. Zhao and co-workers identified a set of lncRNAs that distinguished low-risk from high-risk BC patients [34]. Patients with significantly higher LINC00324 expression and lower PTPRG antisense RNA 1 (PTPRG-AS1) and small nucleolar RNA host gene 17 (SNHG17) expression showed longer overall survival (OS). In another study, high SPRY4 intronic transcript 1 (SPRY4-IT1) expression levels were also associated with poorer prognosis, specifically shorter disease-free survival (DFS) [35].

HOX transcript antisense RNA (HOTAIR) overexpression in BC tissues has been associated with higher invasion and metastatic capacities, and suggested as an OS and progression free-survival (PFS) biomarker [36]. Specifically, in ER^+ BC patients, HOTAIR expression was shown to independently predict the risk of metastasis [37]. Similarly, metastasis-associated lung adenocarcinoma transcript 1's (MALAT1) upregulation was

found in primary BC and its levels were further increased in the respective metastases [38]. Conversely, BC040587 [39], neuroblastoma associated transcript 1 (NBAT1) [40] and eosinophil granule ontogeny transcript (EGOT) [41] were found downregulated in BC samples and associated with poor prognosis. Furthermore, LINC00472 high expression levels in BC tissues associated with less aggressive behavior and more favorable outcome [42].

Predictive biomarkers

LncRNAs have been suggested as valuable predictive biomarkers. Indeed, BC anti-estrogen resistance 4 (BCAR4) overexpression has been shown to predict tamoxifen resistance [43]. On the other hand, lincRNAs LINC00160 and LINC01016 were both found highly overexpressed in ER^+ tumors compared to ER^- tumors and normal tissues, being significantly associated with longer OS of luminal A BC [44]. Interestingly, these lincRNAs may identify patients that respond to ET, functioning as robust predictive biomarkers for ER activity.

Besides ET resistance, progression or recurrence due to resistance to trastuzumab or other commonly used therapeutic approaches, such as chemotherapy and radiotherapy, also constitute a significant clinical challenge. LncRNA activated by TGF-β (ATB) has been associated with trastuzumab resistance in BC patients [45]. Conversely, lncRNA colon cancer associated transcript 2 (CCAT2) overexpression identified a subset of BC patients that might not benefit from Cyclophosphamide, Methotrexate and Fluorouracil (CMF) based adjuvant chemotherapy [46]. Finally, Chen et al. [47] demonstrated that overexpression of lincRNA Regulator of Reprogramming (ROR) is associated chemotherapy tolerance.

MicroRNAs (miRNAs)

MiRNAs are endogenous, small non-coding single-stranded RNAs with an approximate length of 22 nt, encoded by various genomic regions in either sense or antisense

orientation [48]. MiRNAs are critical for a wide range of biological processes exerting a finely tuned regulation of gene expression at posttranscriptional level [49].

MiRNAs in breast cancer

MiRNA dysregulation in cancer was first reported in 2002 [50]. Since then, the emergence of miRNAs has been one of the defining developments in cancer biology with several studies demonstrating a differential miRNA expression profile and global miRNA downregulation in human malignancies compared with paired normal tissues. Indeed, aberrant miRNA expression in human tumors is not just a casual association, as it exerts a causal role at different steps of the tumorigenic process. Some of the miRNAs that will be mentioned here have already been associated with several hallmarks of cancer [3,51,52] (Figure 1).

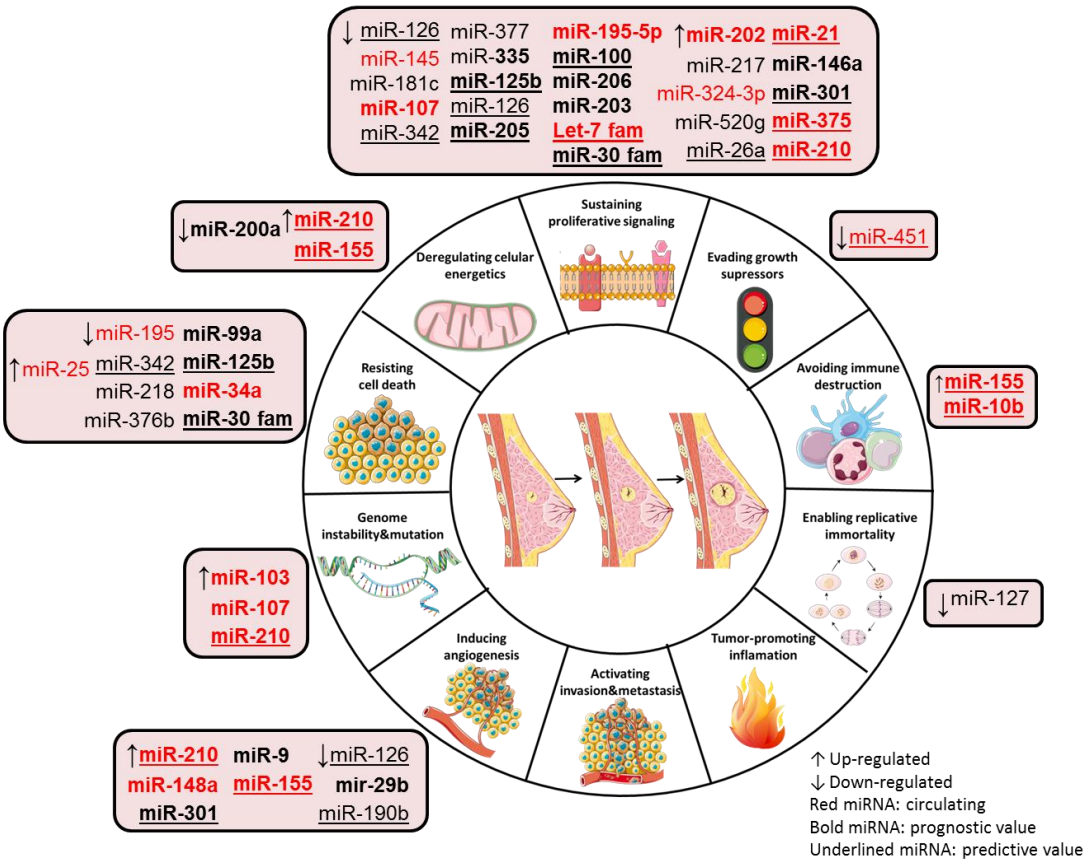

Figure 1. MiRNAs as key regulators of BC hallmarks. Expression of miRNAs (↑up-regulated and ↓ down-regulated) grouped according to their function in the hallmarks of breast cancer: circulating miRNAs (red) and non-circulating miRNAs with prognostic (bold) and predictive (underlined) value. **Abbreviations:** miR – microRNA; fam – family.

MiRNAs might be classified into oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs, depending on their targets. OncomiRs act by repressing the expression of tumor suppressor genes and are frequently upregulated in cancer. Tumor suppressor miRNAs act by targeting oncogenes and are frequently downregulated in cancer. However, this miRNA categorization may be inaccurate, as many studies have shown that miRNAs may present a dual function, with oncogenic or tumor suppressive properties based on tumor type and cellular context [53]. Furthermore, some miRNAs are consistently up- or down-regulated in tumor specimens, whereas other, such as miR-221 and miR-10b, exhibit a more irregular pattern of expression [54]. MiR-10b was found downregulated in all BCs from metastasis-free patients, but elevated miR-10b levels were found in primary tumors from patients harboring metastasis, suggesting that miR-10b might be differentially deregulated along tumor progression [55]. Volinia et al. [56] studied this change in miRNA expression along cancer progression and found that let-7d, miR-210 and miR-221 were downregulated in the ductal carcinoma *in situ* compared to normal breast tissue, but it was found to be upregulated in the transition to invasive carcinoma, featuring an expression reversal along the cancer progression path.

Diagnostic biomarkers

Each tumor type has a distinct miRNA signature that distinguishes it from normal tissues and other cancer types [15]. In 2005, Iorio et al. [57] identified a 13-miRNA signature that could discriminate BC from normal breast tissues with perfect accuracy. Among the differentially expressed miRNAs, the most consistently dysregulated were miR-125b and miR-145 (downregulated), and miR-21 (up-regulated). Since then, many studies have looked at specific miRNAs dysregulated in BC with a diagnostic purpose.

In addition to studies of miRNA expression patterns in tissues, expression profiling studies of miRNAs in bodily fluids have been performed, to investigate whether bodily fluids could be used to differentiate BC patients from healthy individuals. In this context, Heneghan et

al. [58] found significantly higher levels of miR-195 and let-7a in the blood of BC patients compared to healthy controls, detecting BC with high sensitivity and specificity. Several studies have also highlighted differences in the profiles of serum and plasma miRNAs in cancer compared to healthy individuals. MiR-222, for example, was significantly increased in the serum of BC patients [59], while higher miR-205 levels have been found in the sera of healthy individuals compared to BC patients [60]. Furthermore, Zhao et al. found that miR-195 was downregulated in the plasma of BC patients compared to healthy subjects [61].

MiRNA profiles show better diagnostic performance as well as increased sensitivity than individual miRNAs, for BC detection. Hu et al. identified a 4-miRNA signature with increased concentrations in the serum of BC patients that could distinguish BC patients from healthy individuals with 92.1% and 93.4% sensitivity and specificity, respectively [62]. More recently, Zhang and co-workers have found a 3-miRNA signature in serum as a diagnostic biomarker for non-invasive early detection of BC [63], whereas Ng et al. reported that the combination of miR-145 and miR-451 levels in plasma may discriminate normal individuals from BC patients, both at early and advanced stages of disease [64]. Finally, Cuk et al. have also found a panel of deregulated plasma miRNAs that were elevated in women with benign and stage I or II BC, that might be attractive candidates for early BC detection [65].

Table 1 summarizes these and others non-circulating and circulating miRNAs already described and validated in large cohorts for BC diagnosis.

Table 1. Non-circulating and circulating miRNAs for BC diagnosis.

	miRNAs	Sample	Validation techniques	Samples size	Sensitivity	Specificity	AUC	Refs.
Non-circulating miRNAs	↑ miR-23a	BC tissues	qRT-PCR	76BC *vs*. 36 benign *vs*. 36N	0.829	0.100	0.915	[66]
	↑ miR-155, -21, -184, -518b, -572, -601, -622 ↓ miR-125b		TaqMan qRT-PCR	24BC *vs*. 6N	.	.	.	[67]
	↑miR-660-5p ↓miR-99b-5p, -574-3p, -769-5p		SYBR Green qRT-PCR	56BC *vs*. 9N 60BC *vs*. 11N	-	-	-	[68]
Circulating miRNAs	↑miR-222	Serum	qRT-PCR	50BC *vs*. 50N	0.74	0.60	0.671	[59]
	↑miR-16, -25, -222, -324-3p		TaqMan qRT-PCR	76BC *vs*. 76N	0.921	0.934	0.928	[62]
	↑miR-145, -155, -382		qRT-PCR	61BC *vs*. 10N	0.976	0.100	0.988	[69]
	↓miR-205		qRT-PCR	58BC *vs*. 93N	0.862	0.828	0.84	[60]
	↑miR-199a, -29c, -424		SdM-RT-PCR	76BC *vs*. 52N	0.776	0.846	0.901	[63]
	↑ miR-92a, miR-133a		qRT-PCR	132BC *vs*. 101N	-	-	0.91	[70]
	↓miR-200c	Whole blood	qRT-PCR	57BC *vs*. 20N	0.90	0.702	0.79	[71]
	↓miR-145 ↑miR-451	Plasma	TaqMan qRT-PCR	70BC *vs*.50N	0.900	0.920	0.931	[64]
	↑miR-127-3p, -148b, -376a, -376c, -409-3p, -652, -801		TaqMan qRT-PCR	120BC *vs*. 60N	0.800	0.720	0.81	[65]
	↓miR-195		SYBR Green qRT-PCR	210BC *vs*. 102N	0.69	0.892	0.859	[61]
	↑miR-16, -148a, -19b, -22a ↓Let-7d, let-7i, miR-103, -107		qRT-PCR	108BC *vs*. 88N	0.91	0.49	0.81	[72]
	↑miR-505–5p ↑miR- 96–5p		qRT-PCR	114BC vs. 116N	-	-	0.72 0.72	[73]

↑Up-regulated ↓Down-regulated

Abbrevations: N – normal; SdM- Serum-direct Multiplex.

Despite the identification of non-circulating and circulating miRNAs aberrantly expressed in BC, discrepancies remain among the different miRNA signatures reported, probably due to differences in clinicopathological variables and the intrinsic heterogeneity of BC. Therefore, an attempt has been made to develop miRNA signatures that might reflect distinct histopathological features of BC.

Indeed, altered miRNAs levels that predict ER, PR and HER2 receptor status have already been identified (Table 2). Lowery et al. identified a 15-miRNA predictive signature corresponding to the expression of ER, PR, and HER2 receptor status [74]. Recently, Cizeron-Clairac and co-workers found that 20 miRNAs were significantly deregulated in ER^+ compared to ER^- BCs [75]. Up-regulation of miR-1244 and downregulation of miR-30e were specific of ER^- tumors, whereas downregulation of miR-18a, miR-18b and miR-654-3p and up-regulation of miR-342-5p and miR-190b were specific of ER^+ tumors.

Table 2. MiRNAs which increased expression predicts for ER, PR and HER2 receptor status in BC.

ER status		**Refs.**
ER^+	miR-342, -217, -190b, -218, -342-5p	74-76
ER^-	miR-299-3p, -190, -135b, **-182**, **-21**, -30e, -1244 , **-10b**, **-375**	58,74,75,77,78
PR status		
PR^+	miR-520f-520c, -377, **-155**	74,79
PR^-	miR-520g, -527-518a, **-182**, **-10b**, **-375**, **-21**	74,77,78
HER2 status		
$HER2^+$	miR-520d, -376b, -146a-5p, **-375**	74,80
$HER2^-$	miR-181c, **-122**	74,78

Circulating miRNAS are represented in bold.

Circulating miRNAs were also found to correlate with ER, PR and HER2 status in several studies. For example, higher levels of circulating miR-182 [77], miR-21 and miR-10b [58] have been correlated with ER/PR^- tumors. Furthermore, miR-155 expression levels were higher in sera of women with hormone-sensitive BCs [79]. Moreover, higher levels of circulating miR-375 were associated with ER/PR^- and $HER2^+$ tumors, whereas higher levels of circulating miR-122 associated with $HER2^-$ tumors [78].

Several specific miRNA expression profiles have also been associated with BC molecular subtypes. Iorio et al. identified a distinct miRNA signature in luminal BC, with overexpression of miR-191 and miR-26 and downregulation of miR-206 [57]. Likewise, miRNAs might differentiate between basal and luminal tumor subtypes in an independent data set [81]. In an attempt to capture the heterogeneity of Luminal A and Luminal B BCs, Endo et al. compared the expression profiles of miRNAs in ER^+ tissues between $ER^{high}/Ki67^{low}$ tumors and $ER^{low}/Ki67^{high}$ tumors [82]. They found that six miRNAs (let-7a,

miR-15a, miR-26a, miR-34a, miR-193b and miR-342-3p) were upregulated and a single miRNA was downregulated (miR-1290) in $ER^{high}/Ki67^{low}$ tumors [82].

Prognostic biomarkers

MiRNAs have been correlated with clinical and pathological features that associate with prognosis in different tumor types and subgroups of BC patients [83,84]. The search for prognostic biomarkers is a continuous and fundamental work in progress, since patients at higher risk may require differential therapeutic interventions.

One of the main reasons for the BC associated mortality is metastization [85], a complex process that allows the primary tumor cells to spread to the neighboring as well as to distant parts of the organism. MiRNAs appear to be involved in the phenotypic changes associated with metastasis formation, such as epithelial-mesenchymal transition, as well as with the presence of circulating tumor cells, which correlate with metastatic spread [86]. MiRNAs may act either as promotors of BC metastasis or as metastasis suppressors. Metastasis promoters include miR-9 [87], miR-10b [55,88], miR-21 [89], miR-29a [90], miR-155 [91], miR-520c [92], miR-373 [88,92], miR-214 [93], miR-301 [94] and miR-548j [95], whereas metastasis suppressors include miR-17/20 [96], miR-126 [97], miR-193b [98], miR-206 [99], miR-335 [100], miR-448 [101], miR-601 [102], miR-138 [103], miR-515-5p [104], miR-203 [105], miR-200 family and miR-205 [106]. These specific miRNAs might serve as valuable biomarkers for predicting metastasis and tumor recurrence, which determine the unfavorable prognosis of BC patients. All these miRNAs were validated in tumor tissues and/or bodily fluids from BC patients and are depicted in Figure 2.

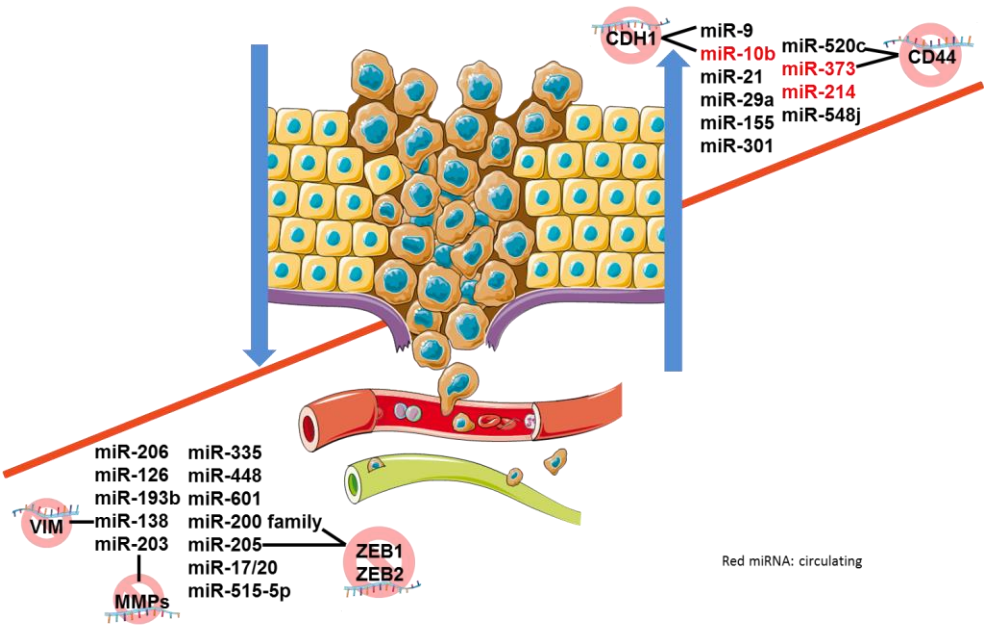

Figure 2. MiRNAs and breast cancer metastasis. MiRNAs are crucial in metastatic spreading, acting either as oncogenes, typically up-regulated, or as tumor suppressor genes, typically down-regulated. Circulating (red), non-circulating miRNAs and examples of targets. **Abbreviations:** miR – microRNA; CD - cluster of differentiation; ZEB - Zinc Finger E-Box Binding Homeobox 1; MMPs - Matrix metalloproteinases; VIM – vimentin; CDH1 – Cadherin 1.

MiRNAs have also been associated with other clinical and pathological features that influence BC patients' prognosis. MiR-21, aside from being a driver of metastasis, has been known to create a pro-tumorigenic environment by targeting numerous tumor suppressor genes, and its overexpression was correlated with advanced tumor stage and poor OS and DFS in BC patients [107,108]. Several studies have independently associated miR-210 with BC development and its expression levels were correlated with tumor aggressiveness and poor prognosis [109,110]. Moreover, some miRNAs have been associated with a good prognosis, such as the miR-30 family, that has been identified as an individual favorable prognostic marker in several studies [111-113]. Other miRNAs, particularly downregulation of the miR-200 family, have also been associated with BC stem cells [114], one of the main obstacles for effective treatment of BC [115].

Some studies have focused on particular subtypes of BC. Bailey et al. evaluated miRNAs expression in ER^+ BC tissues and found that a cluster comprising let-7c and miR-125b was uniformly low in luminal B and lost in a subset of luminal A patients with worse OS,

indicating its potential as biomarker of good outcome in ER^+ luminal A BC patients [116]. Gasparini and co-workers identified a 4-microRNA signature in triple negative BC that allowed for the stratification of those patients into high- and low-risk groups [117]. Up-regulation of miR-493 and miR-155 correlated with better patient outcome, whereas miR-30e and miR-27a downregulation correlated with worse outcome [117].

Interestingly, some miRNAs may differentially influence outcome depending on the characteristics of the tumors. Tuomarila et al. reported that high miR-200c expression independently predicted poor OS in patients with PR^- tumors, whereas low expression independently predicted poor OS in patients with PR^+ tumors [118].

These and other miRNA signatures or single miRNAs that have been associated with prognosis are summarized in Table 3.

Predictive biomarkers

The role of miRNAs as potential predictive biomarkers is also a field of growing interest. When investigating the regulation of miRNAs expression by antiestrogen therapies in human BC specimens using the initial biopsy and comparing it with the surgery specimen after neoadjuvant ET, Maillot and co-workers [146] noticed that some miRNAs that were previously shown overexpressed in tamoxifen-resistant cell lines were up-regulated following ET. These results highlight the utility of considering miRNA expression in understanding ET resistance in BC. Other studies have searched for miRNAs able to predict therapeutic response of BC patients to ET. For instance, Rodriguez-Gonzalez and colleagues [147] have found that miR-30c independently predicted clinical benefit of tamoxifen therapy in patients with advanced BC. On the other hand, Rothe et al. [110] found that miR-210 high level expressions were associated with a higher risk of recurrence in tamoxifen treated patients.

Table 3. MiRNA panels or single miRNAs proposed with a prognostic aim.

	miRNA	Biological sample	Consequences	Refs.
miRNAs associated with positive outcome	**miR-100**	Tumor tissues	↑ OS	119
	miR-29c			120
	miR-181d, -195-5p			80
	miR-128			121
	Let-7b, miR-205		↑ RFS, OS	122
	miR-342-5p			123
	miR-497		↓ TNM, LNM	124
	miR-133a		↑ RFS	125
	miR-30 family		↑ OS, RFS, DFS ↓Metastasis	111-113
	miR-206		↑OS ↓TNM, LNM	126
	miR-601		↓Metastasis ↑MFS	102
	miR-124		↑ OS ↓TNM, LNM	127
	miR-138		↓TNM, LNM	103
	miR-190b		↑MFS, OS	75
	miR-200b		↓ LNM	128
	miR-29b		↑ DFS, OS	129
	miR-27a			130
	miR-374b-5p, -218-5p, -126-3p	TNBC tissues	↑DFS, OS	131
	miR-155 -493		↑ OS	117
	let-7c, miR-99a, -125b	Luminal A BC tissues	↑ OS	116
miRNAs associated with negative outcome	**miR-21**	Tumor tissues	↑ Grade, TNM, LNM, metastasis ↓DFS, RFS, OS	107,108
	miR-210		↓OS, RFS, DFS, MFS ↑Grade	109
	miR-23a		↓RFS	66
	miR-423		↑Metastasis	132
	miR-9		↑ Grade, metastasis, LR	87
	miR-187		↓ DSS, RFS	133
	miR-155		↑TNM, grade, LNM ↓OS	134
	miR-221/222		↓ MFS	135
	miR-421, -486, -503, -720, -1303		↓MFS	136
	miR-375		↑LR	137
	miR-548 family		↑ LNM ↓MFS	95
	miR-146a-5p		↓OS	80
	miR-27b-3p	TNBC tissues	↑ Metastasis ↓DSS	138
	miR-93		↑ LNM, TNM, grade, Ki-67	139
	miR-21, -210, -221		↓DFS, OS	140
	miR-34b			141
	miR-18b, -103, -107, -652	TNBC patients serum	↓ OS, RFS.	142
	miR-200b	BC patients plasma	↓PFS, OS	86
	miR-202	BC patients serum	↓OS	143
	miR-10b-5p		↑TNM, grade, LNM	144
	miR-122		↓MFS, RFS	78
	miR-10b, -34a, -155		↑Metastasis	145

↑Incresead; ↓Decreased

Abbreviations: RFS - Relapse-free survival; TNM - TNM Classification of Malignant Tumours; MFS - Metastasis-free survival; LR - Local recurrence; DSS - Disease-specific survival.

In addition to ET, miRNAs have been involved in responsiveness to other therapies. For instance, high circulating levels of miR-210 have been associated with resistance to anti-HER2 therapy using trastuzumab [148] and miR-100 expression has been positively

correlated with sensitivity to chemotherapy using paclitaxel [119]. The potential role of miRNAs in the prediction of the response to these and other therapies, such as radiotherapy, are summarized in Table 4.

Table 4. MiRNAs involved in therapeutic response (sensitivity/resistance) in BC.

Therapies		Role in response	miRNA	Putative targets	Agent	Biological samples	Refs.
Hormone therapy	Antiestrogens	Sensitivity	**miR-342**	*CCNB1*	Tamoxifen	Cell lines and tumor tissues	149
			miR-26a	*EZH2*		Tumor tissues	150
			miR-30c	*EGFR*			147
			miR-10, -126	-			151
		Resistance	**miR-221/222**	*CDKN1B*	Tamoxifen, fulvestrant	Tumor tissues and cell lines	152,153
			miR-519a	*CDKN1B PTEN*, *RB1*	Tamoxifen	Tumor tissues and cell lines	154
			miR-155	*SOCS6*			131
			miR-210	-		Tumor tissues	110
			miR-301	*FOXF2*, *BBC3*, *PTEN*, *COL2A1*		Tumor tissues, cell lines and xenografts	94
	Aromatase inhibitors	Sensitivity	**miR-125b let-7c**	*ERBB2*	Letrozole, anastrozole	Tumor tissues and cell lines	116
		Resistance	**miR-181a**	*BCL2L11*	Letrozole	Cell lines, xenografts and tumor tissues	155
Antibodies		Sensitivity	**miR-210**	-	Trastuzumab	Cell lines and plasma	148
		Resistance	**miR-21**	*PTEN*	Trastuzumab	Cell lines, xenograft and tumor tissues	156
Chemotherapy		Sensitivity	**miR-451**	*MRP-1*	Doxorubicin	Cell lines and tumor tissues	157
			miR-200c	*MRP-1*			158
			miR-134	*ABCC1*			159
			miR-128	*BMI1*, *ABCC5*	Doxorubicin, Paclitaxel	Cell lines, xenografts and tumor tissues	121
			miR-100	*MTOR*	Paclitaxel	Cell lines, xenografts and luminal tumor tissues	119
			miR-16	*IKBKB*		Cell lines and tumor tissues	160
			miR-621	*FBXO11*	Paclitaxel +Carboplatin	Cell lines, xenografts and tumor tissues	161
		Resistance	**miR-125b**	*BAK1*, *E2F3*	FEC	Cell lines, tumor tissues and serum	162
			miR-141	-	Taxane, Anthracyclines	Cell lines and tumor tissues	163
			miR-221	*CDKN1B*		Plasma	164
			miR-155	*FOXO3a*	Paclitaxel, VP-16, Doxorubicin	Cell lines and tumor tissues	165
Radiotherapy		Sensitivity	**miR-155**	*RAD51*	-	Cell lines and TNBC tissues	166

Abbreviations: *CCNB1* - Cyclin B1; *EZH2* - Enhancer of zeste homolog 2; *EGFR* - epidermal growth factor receptor; *CDKN1B* - Cyclin-Dependent Kinase Inhibitor 1B; *PTEN* - phosphatase and tensin homolog; *RB1* – retinoblastoma 1; *SOCS6* - suppressor of cytokine signaling 6; *FOXF2* - forkhead box F2; *BBC3*- BCL2 binding component 3; *COL2A1* - collagen type II alpha 1; *ERBB2* - Erb-B2 Receptor Tyrosine Kinase 2; *BCL2L11* - Bcl-2-like protein 11; *MRP1* - Multidrug resistance-associated protein 1; *ABCC1* - ATP binding cassette subfamily C member 1; *BMI1* - Bmi1 polycomb ring finger oncogene; *ABCC5* - ATP binding cassette subfamily C member 5; *MTOR* - mechanistic target of rapamycin; *IKBKB* - IκB kinase β; *FBXO11* - F-box protein 11; *BAK1* - BCL2 antagonist/killer 1; *E3F3* - E2F transcription factor 3; *FOXO3a* - Forkhead box O3a; *RAD51* - RAD51 recombinase; FEC - 5-Florouracil, Epirubucin and Cyclophosphamide.

Several clinical trials, summarized in Table 5, are currently ongoing to address the role of miRNAs in diagnosis, prognosis and prediction of response to therapy, aiming at the translation of current knowledge on miRNAs in BC into clinical practice.

Table 5. Ongoing clinical trials aiming at the introduction of miRNAs in clinical practice.

Clinical trial	Patient population	Intervention	Aims	Study start date
NCT00581750 Observational	Patients with lobular carcinoma *in situ*	Tumor profiling	Diagnosis	October 2001
NCT01231386 Observational	Patients undergoing neoadjuvant or adjuvant chemotherapy and HT for locally advanced & inflammatory BC	Tumor profiling Circulating miRNAs	Prognosis Drug sensitivity	October 2014
NCT01722851 Observational	Newly diagnosed BC patients who are scheduled to undergo neoadjuvant chemotherapy/HT and patients who present with disease recurrence or disease progression, and who are commenced on systemic therapies (HT and/or chemotherapy)	Circulating miRNAs	Prognosis Drug sensitivity	September 2013
NCT02656589 Observational	Patients with $HER2^{+}$ advanced stage BC who were treated with Herceptin		Drug sensitivity	June 2015
NCT01598285 Observational	Patients suffering from metastatic BC, treated with bevacizumab			May 2012
NCT01612871 Observational	Patients with metastatic invasive BC or locally advanced BC for which treatment with tamoxifen or anti-aromatase is indicated			June 2012

CONCLUSION

BC is a very heterogeneous disease, and several biological features are routinely used for diagnostic, prognostic and predictive purposes, including histological grade, lymph node status, hormone receptor status, and HER2 status. These factors have been associated with BC patient's survival and clinical outcome following treatment. Nevertheless, some

patients with similar combination of those features follow different clinical paths, demonstrating that the capacity of determining prognosis and predicting therapeutic outcome in BC patients remains limited. Several mRNA-based tests are currently available with the aim of improving BC prognostication, but its use in clinical practice is still limited. New biomarkers are therefore needed to assist in improving BC patient prognostication and monitoring, allowing for a more precise definition of the therapeutic and follow-up strategy in an individual basis.

Based on the studies cited in this review, it is remarkable that ncRNAs are adding an extra dimension to the understanding of BC biology. MiRNAs, in particular, are emerging as promising biomarkers for BC diagnosis (e.g. miR-155 and miR-195), prognosis (e.g. miR-29b and miR-30 family) and prediction of response to therapy (e.g. miR-30c and miR-221). It should be emphasized that miRNAs are easily accessible, affordable, non-invasive tools for personalized management of BC patients, since they circulate stably in bodily fluids. These features allow miRNAs to respond to current clinical needs and provide the opportunity to bypass the problems associated with tumor tissue biopsy. Although some lncRNAs have also shown potential to serve as BC biomarkers, the stability and origin of circulating lncRNAs remain largely unknown, and additional studies are required to support a definitive clinical application. Regarding tRNAs, many questions also remain unanswered, such as the origin and its physiological role.

When reviewing the data from several studies, widespread inconsistencies across them are found. The cause might be attributable to differences in sample type, with some studies using plasma or serum and other using whole blood, differences in technology platforms used for miRNA profiling, such as next-generation sequencing (NGS) or real time reverse transcription polymerase chain reaction, differences in the choice of pre- or –post-operative samples, as well as from the choice of different genes for data normalization. These discrepancies among reported signatures highlight the need to

standardize experimental conditions for circulating miRNAs studies, as well as the need to validate these findings in additional independent cohorts as well as preclinical/clinical verification studies, before the clinical utility of circulating miRNAs may be established.

In conclusion, the emergence of ncRNA classes as possible BC biomarkers, mainly miRNAs, shows great potential to foster precision medicine in BC, although its application in clinical routine is still a long term goal.

Abbrevations:

BC – breast cancer

BCAR4 - BC anti-estrogen resistance 4

CCAT2 - lncRNA colon cancer associated transcript 2

CMF - Cyclophosphamide, Methotrexate and Fluorouracil

DFS – disease-free survival

EGOT - eosinophil granule ontogeny transcript

ER – estrogen receptor

PR – progesterone receptor

HER2 - human epidermal growth factor receptor 2

ET – endrocrine therapy

HOTAIR - HOX transcript antisense RNA

HT – Hormone-therapies

LincRNAs - long intergenic non-coding RNAs

lncRNAs – long noncoding RNAs

LNM - lymph node metastasis

MALAT1 - metastasis-associated lung adenocarcinoma transcript 1

MiRNAs – microRNAs

mRNA – messenger RNA

NBAT1 - neuroblastoma associated transcript 1

ncRNAs – noncoding RNAs

OncomiRs - oncogenic miRNAs

OS – overall survival

PFS - progression free-survival

PTPRG-AS1- PTPRG antisense RNA 1

ROR - Regulator of Reprogramming

rRNAs - ribosomal RNAs

SPRY4-IT1 - SPRY4 intronic transcript 1

SNHG17 - small nucleolar RNA host gene 17

snoRNAs - small nucleolar RNAs

tRNAs – transfer RNAs

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Ethics approval and consent to participate

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Consent for publication

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Competing interests

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MA and SS revised the literature and wrote the paper. RH and CJ revised the manuscript. All the authors read and approved the final manuscript

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Appendix VI. MiRNAs with fold variation values higher than 1 in the global expression assay.

LumA Rec *vs*. LumA NRec		LumB Rec *vs*. LumB NRec		Lum Rec *vs*. Lum NRec	
microRNA	**Fold Change**	**microRNA**	**Fold Change**	**microRNA**	**Fold Change**
miR-196a-5p [2]	2,1281	**miR-9-5p** [1]	2,5978	**miR-9-5p** [1]	1,4448
miR-181b-5p	-1,0119	**miR-210-3p** [1]	1,7178	**miR-149-3p** [1]	1,23995
miR-130a-3p [1]	-1,0519	**miR-182-5p** [2]	1,6028	**miR-126-3p**	-1,0909
miR-29b-3p	-1,1169	**miR-7-5p** [1]	1,3978	**miR-1**	-1,1352
let-7b-5p	-1,1269	**miR-200c-3p**	1,2778	**miR-148a-3p**	-1,1419
let-7i-5p	-1,1369	**miR-31-5p** [1]	1,0928	**miR-30d-5p**	-1,2139
miR-106b-5p	-1,1419	**miR-221-3p**	1,0128	**miR-181a-5p** [2]	-1,4322
miR-132-3p [1]	-1,1519	**miR-125b-5p**	-1,0172	**miR-200a-3p**	-1,5732
miR-26b-5p	-1,1619	**miR-146a-5p**	-1,0372	**miR-205-5p** [2]	-2,3252
miR-19b-3p	-1,1769	**miR-181a-5p** [2]	-1,0622		
miR-192-5p [1]	-1,1969	**miR-205-5p** [2]	-1,1172		
let-7g-5p	-1,2019	**miR-1** [1]	-1,1472		
miR-16-5p	-1,2319	**miR-10b-5p**	-1,4022		
miR-15a-5p	-1,2619				
miR-106a-5p	-1,2669				
miR-20a-5p	-1,2769				
let-7a-5p	-1,3019				
miR-21-5p	-1,3169				
miR-214-3p	-1,3569				
miR-93-5p	-1,4119				
let-7f-5p	-1,4369				
miR-222-3p	-1,4419				
miR-200c-3p	-1,4719				
miR-155-5p	-1,5119				
let-7e-5p	-1,5119				
let-7d-5p	-1,5619				
miR-148a-3p	-1,6369				
miR-181a-5p [2]	-1,6519				
miR-23b-3p	-1,7569				
miR-23a-3p	-1,8069				
miR-19a-3p	-1,8519				
miR-1 [1]	-1,8869				
miR-221-3p	-1,9319				
miR-195-5p	-1,9369				
miR-18a-5p [1]	-1,9919				
miR-30c-5p [2]	-2,0419				
miR-182-5p [2]	-2,1119				
miR-186-5p [1]	-2,1319				
miR-141-3p	-2,1619				
miR-17-5p [1]	-2,1919				
miR-30d-5p	-2,2769				
miR-30b-5p [2]	-2,4819				
miR-101-3p	-2,5319				
miR-200b-3p [2]	-3,0019				
miR-92b-3p [1]	-3,1069				
miR-200a-3p	-3,2169				
miR-205-5p [2]	-4,1269				

[1] Cps higher than 30 [2] miRNAs chosen for further validation
Abbreviations: Lum – Luminal; Rec – Recurrent.

Appendix VII. Univariable cox regression models assessing the association between clinicopathological features and clinical outcome.

Model	Outcome	Variable	HR (95% CI)	*p*-value
Univariable Analysis	**ERFS**	**HER2 *status*** Negative Positive	1 3.459 (1.352-8.851)	0.010
		Ki-67 index <15% >15%	1 5.823 (2.254-15.042)	<0.001
		Molecular subtype Luminal A Luminal B	1 5.109 (1.511-17.271)	0.009
		Grade G1&G2 G3	1 2.689 (1.114-6.492)	0.028
	DFS	**HER2 *status*** Negative Positive	1 3.325 (1.570-7.041)	0.002
		Ki-67 index <15% >15%	1 2.475 (1.243-4.926)	0.010
		Grade G1&G2 G3	1 2.208 (1.156-4.218)	0.016
	DMFS	**HER2 *status*** Negative Positive	3.856 (1.796-8.278)	0.001
		Ki-67 index <15% >15%	2.271 (1.090-4.734)	0.029
		Grade G1&G2 G3	2.247 (1.133-4.456)	0.020

Abbreviations: ERFS – endocrine resistance-free survival; DFS – disease-free survival; DMFS – distant metastasis-free survival; HER2 – human epidermal growth factor 2 receptor; G – grade; HR – Hazard ratio; CI – confidence interval.